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Investigation of Chlorine Treatment to Enhance DNA-Based Detection of the *Bacillus anthracis* Spore

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FOR THE DIRECTOR

//signed//
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There is a crucial need for technology that allows rapid and reliable detection of waterborne pathogens. Pathogenic organisms such as *Vibrio cholerae, Salmonella* sp., *Shigella* sp., enteropathogenic *Escherichia coli, Campylobacter jejuni, Cryptosporidium parvum*, and *Giardia lamblia* have long been known to pose a public health threat to drinking water supplies. More recently, there has been concern that pathogenic microorganisms such as *Bacillus anthracis* spores could deliberately be introduced into a water supply as an act of bioterrorism. *Bacillus anthracis*, the causative agent of anthrax, ranks high on the list of bioterrorism agents that pose a threat to drinking water supplies. Dormant spores are capable of surviving in both aquatic and terrestrial environments for long periods.

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1. INTRODUCTION

There is a crucial need for technology that allows rapid and reliable detection of waterborne pathogens. Pathogenic organisms such as *Vibrio cholerae, Salmonella* sp., *Shigella* sp., enteropathogenic *Escherichia coli, Campylobacter jejuni*, *Cryptosporidium parvum*, and *Giardia lamblia* have long been known to pose a public health threat to drinking water supplies. More recently, there has been concern that pathogenic microorganisms such as *Bacillus anthracis* spores could deliberately be introduced into a water supply as an act of bioterrorism. *Bacillus anthracis,* the causative agent of anthrax, ranks high on the list of bioterrorism agents that pose a threat to drinking water supplies. Dormant spores are capable of surviving in both aquatic and terrestrial environments for long periods.

While molecular-based systems such as Polymerase Chain Reaction (PCR) offer great potential for effective pathogen detection, intricacies of these methods can pose limitations to practical application. One of the primary obstacles for PCR-based detection of pathogens is gaining access to the cellular DNA from a wide range of microbial types. While the DNA of many microorganisms can be released from the cells with relative ease, gaining access to the DNA of bacterial spores, such as those produced by *B. anthracis*, is extremely difficult. Since the permeabilization of spores presents one of the most formidable cellular barriers to rapid DNA-based detection, the disruption of anthrax spores was the primary focus of this research. This effort involved investigating processes that would enhance the permeability of microorganisms for DNA-based detection. The potential effectiveness of chlorine as well as antimicrobial peptides to disrupt spores prior to DNA-based detection was investigated.

2. MICROSCOPIC EXAMINATION OF *B. ANTHRACIS* EXPOSED TO CHLORINE.

Chlorine is a strong oxidizing agent with a broad spectrum of antimicrobial activity. Since chlorine is well know for its sporicidal disinfectant properties. The feasibility of using chlorine for the rapid disruption of spores prior to PCR-based detection was investigated. The disruptive effects of chlorine on dormant spores and vegetative cells was initially assessed by examining the microscopic appearance of the organisms following chlorine treatment.

2.1. Microscopic Appearance of Chlorinated *B. anthracis* Spores.

Effects of chlorine on dormant spores incubated at ambient temperature, 50^{o}C, or 85^{o}C was investigated. Spore morphology following chlorine treatment was determined by phase-contrast microscopy.

Results. The temperature at which the chlorination was performed had a profound effect on the extent of spore disruption and, consequently, the microscopic appearance of the spores. When the chlorination of dormant spores was performed at ambient temperature, chlorine concentrations ranging from 10 to 100 ppm produced no observable changes in the microscopic appearance of the spores; the spores remained refractile and intact (data not shown). However, when the exposure temperature was increased to 50 or 85^{o}C, chlorine rapidly affected the appearance of the spore coat and the refractivity of the spores. The changes in the microscopic

appearance of spores following chlorine treatment at 85°C for 1 minute, 85°C for 5 minutes, or 50°C for 5 minutes are presented in Tables 1, 2, and 3, respectively.

TABLE 1. Microscopic Appearance of Spores Treated with Chlorine at 85°C for 1 Minute

Chlorine Concentration	Appearance of Spores Following Chlorine Treatment
0 ppm	Bright, refractive spore core
10 ppm	Slight thickening of spore coat
25 ppm	Thickened spore coat, slight loss of spore core refractivity
50 ppm	Thickened spore coat, slight reduction in spore core refractivity
75 ppm	Spore core dark with some light areas
100 ppm	All spore cores dark

TABLE 2. Microscopic Appearance of Spores Treated with Chlorine at 85°C for 5 Minutes

Chlorine Concentration	Appearance of Spores Following Chlorine Treatment
0 ppm	Bright, refractive spore core
10 ppm	Thickened spore coat, slight loss of core refractivity
25 ppm	Darkened spore core
50 ppm	Many spores disrupted with remnants of spore coat remaining
75 ppm	Most spores disrupted with spore remnants and debris remaining

TABLE 3. Microscopic Appearance of Spores Treated with Chlorine at 50°C for 5 Minutes

Chlorine Concentration	Appearance of Spores Following Chlorine Treatment
0 ppm	Bright, refractive spore core
10 ppm	Some spores slightly thickened coat
25 ppm	Thickened spore coat; spore core refractive
50 ppm	Thickened spore coat; loss of spore core refractivity
75 ppm	Most spore cores dark

2.2. Microscopic Appearance of Chlorinated *B. anthracis* Cells.

To examine the effects of chlorine on *B. anthracis* cells, 1.0-ml suspensions were harvested and suspended in 1.0-ml distilled water. The suspensions (10 μl) were added to 100-μl reaction mixes that contained 50-, 100-, or 200-ppm chlorine. The samples were heated at 85°C for 5 minutes and the microscopic appearance of the cells was examined by phase-contrast microscopy.

Results. *B. anthracis* rods were rapidly disrupted with chlorine concentrations ranging from 50 to 200 ppm when heated at 85°C for 5 minutes. The results are presented in TABLE 4.

TABLE 4. Microscopic Appearance of *B. anthracis* Cells Chlorinated with 0-, 50-, 100-, or 200-ppm Chlorine at 85°C for 5 Minutes

Chlorine Concentration	Appearance of Rods Following Chlorine Treatment at 85°C
0 ppm	Dark, intact cells
50 ppm	Cell density varied from light gray to dark
100 ppm	Cell wall appeared intact, portions cell interior very light, granular, and much less dense.
200 ppm	Few intact cells; perhaps most cells lysed

3. PCR METHODOLOGY AND STANDARD PCR CONDITIONS.

3.1. PCR Constituents. PCR was conducting using the GeneAmp PCR Reagent Kit with AmpliTaq DNA polymerase (Perkin Elmer). PCR constituents are listed in TABLE 5. PCR amplification of Lambda bacteriophage DNA for generation of a 500-bp product was conducted using the following primers:

5'-GAT GAG TTC GTG TCC GTA CAA CTG G-3' (forward),
5'-GGT TAT CGA AAT CAG CCA CAG CGC C-3' (reverse)

PCR amplification of *B. anthracis* DNA was accomplished by targeting of a sequence of the *capB* region of plasmid X02 to generate a 623-bp product using the following primers:

5'-ACA ACT GGT ACA TCT GCG CG-3' (forward),
5'-GAT GAG GGA TCA TTC GCT GC-3' (reverse).

TABLE 5. PCR Master Mix (50-μl Total Volume)

PCR Components (Concentration)	Volume (μl)	Final Concentration
De-ionized H_2O	for 50-μl total	
Buffer (10x PCR buffer II, Perkin Elmer)	5.0	1x
dNTP mix (1.25 mM)	8.0	200 μM
25% Tween 20 (25%)	4.0	2%
Forward Primer		0.2 μM
Reverse Primer		0.2 μM
$MgCl_2$ (25 mM)	4.0	2 mM
AmpliTaq DNA polymerase (5 units/μl)	0.25	1.25 unit/50 μl
DNA Template Sample	10	

3.2. PCR Thermal Cycle Conditions. PCR trials were performed using the model 9600 thermal cycler (ABI Prism). A two-step PCR program with 35 cycles was employed which for each PCR cycle involved a denaturing step and a step that combined annealing and extension. Thermal cycle conditions of the two-step PCR (designated as program 16) are presented in TABLE 6.

TABLE 6. PCR Program 16 Thermal Cycle Conditions

Temperature	Time (PCR Action)	
94°C	1 minute (initial denaturing)	
94°C	15 seconds (step 1 for denaturing)	35 cycles
68°C	1 minute (step 2 for annealing and extension)	
72°C	10 minute (final extension)	

3.3. Designation of PCR Product Quantity. PCR product of experimental trials was fractionated through 1% agarose at 100 volts for 45 minutes, stained with ethidium bromide, and visualized by UV sub-stage illumination. PCR product for a given sample was reported as being present or absent as determined by visual inspection of stained agarose gels. In addition, quantity of PCR product was designated on the basis of DNA band size. The presence of a DNA band as visualized in a stained gel was indicated by "+" and the absence of a band was indicated by "--". Quantity of PCR product was designated on the basis of DNA band width. Criteria for PCR product quantity designations are presented in TABLE 7.

TABLE 7. Designation of PCR Product Quantity as Indicated by DNA Appearance on Stained Agarose Gel

DNA Quantity Designation	Criterion for Designation
+++	>20-mm band thickness
++	10- to 20-mm band thickness
+	<10-mm band thickness
trace	trace of DNA visible
--	no DNA visible

4. PCR-BASED DETECTION OF THE CHLORINATED *B. ANTHRACIS* TARGET ORGANISM.

4.1. PCR of Chlorinated Spores. The following experiments were performed to determine whether the treatment of spores with chlorine could provide a rapid means of disrupting *B. anthracis* spores prior to PCR-based detection.

4.1.1. PCR of Spores Exposed to 0-, 10-, 25-, 50- or 75-ppm Chlorine at 85°C for 1 Minute.

Spores (10^4 in a 10-µl volume) were chlorinated with 0-, 10-, 25-, 50-, or 75-ppm chlorine. Samples were heated at 85°C for 1 minute and the chlorine was neutralized with 10-mM sodium thiosulfate. The total spore volume was assayed directly using PCR.

RESULTS. The amplification of *B. anthracis* spore DNA was substantially inhibited following chlorine treatment. PCR inhibition occurred when the spores were treated with either 10-, 25-, 50- or 75-ppm chlorine. The control containing sodium thiosulfate in the absence of chlorine did not inhibit PCR (TABLE 8).

TABLE 8. PCR Amplification of Spores Chlorinated at 85°C for 1 Minute

Chlorine Concentration for Spore Treatment at 85°C	Amplification Signal
0 ppm (25°C spore control)	++
0 ppm (85°C spore control)	++
0 ppm (85°C control + thiosulfate)	++
10 ppm	+
25 ppm	--
50 ppm	trace
75 ppm	trace

4.1.2. PCR of Spores Exposed to 0-, 10-, 25-, 50- and 75-ppm Chlorine at 50°C for 5 Minutes.

The chlorination of spores at 50°C for 5 minutes was also highly effective for disrupting the spore coat as determined by microscopic examination. This experiment was performed to determine whether the chlorination of spores at 50°C could enhance the PCR-based detection of dormant spores, yet minimize the formation of PCR inhibitors. In this experiment, spores (2.8 x 10^5 in a 10-µl volume) were exposed to 0-, 10-, 25-, 50- or 75-ppm chlorine at 50°C for 5 minutes and then neutralized with sodium thiosulfate. The spores were used directly for PCR. The PCR amplification reactions of the chlorinated spores were run in the presence and absence of Tween 20.

RESULTS. While there was good PCR amplification for the samples containing non-chlorinated spores, PCR was inhibited in all of the chlorinated spore suspensions. The incorporation of Tween 20 in the PCR reaction mix slightly enhanced the amplification in the 0-ppm control, as well as the 10- and 50-ppm chlorinated spore samples (TABLE 9).

TABLE 9. PCR Amplification of Spores Chlorinated at 50°C for 5 Minutes

Sample Treatment	Tween 20 in Master Mix	No Tween 20 in Mix
0 ppm 25°C (control)	++	++
0 ppm 50°C (control)	++	++
10 ppm	trace	--
25 ppm	--	--
50 ppm	trace	--
75 ppm	--	--
10^5 spores (control)	++	++
10^4 spores (control)	++	++

4.1.3. PCR of Chlorinated Spores with a Range of Spore Densities.

This experiment was performed to determine whether higher numbers of chlorinated spores produce greater levels of PCR inhibition. In this experiment, 10^8, 10^7, and 10^6 spores were treated with 25-ppm chlorine at 50°C for 5 minutes. The chlorine was neutralized with sodium thiosulfate and 5 µl of the chlorinated spore suspension was used as template for PCR. The final spore numbers in the PCR reaction mixes were 7.5×10^5, 7.5×10^4, and 7.5×10^3.

In addition, a duplicate set of reactions was prepared to compare the PCR amplification of spores that had been chlorinated in either small reaction volumes (10 µl) or large reaction volumes (80 µl). Both of these chlorination strategies have been performed in past experiments.

Results. In this experiment, no amplification was obtained in any of the chlorinated samples. For the non-chlorinated samples, the strength of the signals corresponded to the number

of organisms added to the PCR mixes. Similar results were obtained when the chlorination reactions were performed in either a small or large volume (Tables 10 and 11).

TABLE 10. PCR Following the Chlorination of Spores in Small Reaction Volume (10 μl)

Chlorine Concentration	Number of Spores		
	10^5	10^4	10^3
0 ppm	++	+	<+
25 ppm	--	--	--

TABLE 11. PCR Following the Chlorination of Spores in Large Reaction Volume (80 μl)

Chlorine Concentration	Number of Spores		
	10^5	10^4	10^3
0 ppm	++	+	--
25 ppm	--	--	--

4.1.4. Spores Exposured to Chlorine at 37°C for 5 Minutes.

This experiment was performed to determine whether the chlorination of spores at a lower treatment temperature (37°C) could disrupt the spore coat, yet prevent the production of PCR inhibitors. In this experiment, 10^5 spores were exposed to 0-, 10-, 25-, 50- or 100-ppm chlorine at 37°C for 5 minutes. Chlorine was neutralized with 10-mM sodium thiosulfate, and spores were used directly for PCR.

Results. For the dormant spore suspensions which were treated with chlorine at 37°C, the PCR inhibition increased as the concentration of chlorine increased (TABLE 12).

TABLE 12. Amplification of DNA from Spores Chlorination at 37°C for 5 Minutes

Chlorine Concentration (ppm)				
0	10	25	50	100
+++	++	+	Trace	--

4.2. Chlorination of *B.* anthracis Cells. A series of experiments was performed to determine whether the chlorination of *B. anthracis* cells also prohibits the PCR-based detection of the organism. Since the physiology of the spore surface differs significantly from that of the cell, it is possible that the chlorine treatment of the cells would not yield inhibitors that prevent PCR-based detection.

4.2.1. Chlorine Consumption by Nutrient Broth.

In several chlorination experiments using cells, inhibition of PCR was not observed and microscopic appearance of the cells was not altered by chlorine treatment. In these experiments, the cells had not been washed and the residual culture medium was found to consume the chlorine. This experiment was performed to determine the number of washes that are needed to efficiently remove the nutrient broth and prevent the consumption of chlorine.

Results. Two washes were found to be effective at removing residual nutrient broth for the chlorination studies (FIGURE 1).

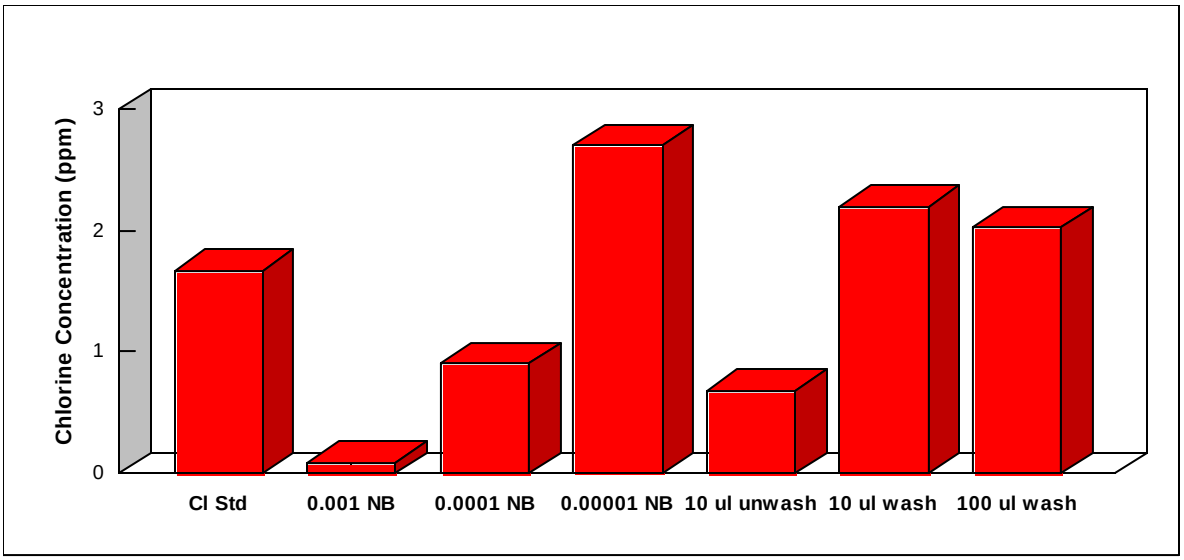

FIGURE 1. Chlorine Consumption by Nutrient Broth and its Removal by Washing. Chlorine consumption was tested in the presence of 0.1, 0.01, and 0.001% Nutrient Broth. In addition, unwashed cells (10% inoculum) and cellss (10% inoculum) which were washed twice were tested for their capacity to consume chlorine.

4.2.2. PCR of Cells Exposed to Chlorine.

This experiment was performed to determine whether the chlorination of *B. anthracis* cells was inhibitory to PCR. Cells (1×10^5) were exposed to 0-, 25-, 50-, or 75-ppm chlorine at either 50^oC for 5 minutes or 80^oC for 1 minute.

Results. As with anthrax spores, the chlorination of *B. anthracis* cells interfered with PCR (TABLE 13). The extent of PCR inhibition increased as the chlorine concentration increased. Cells heated at 50^oC or 80^oC in the absence of chlorine yielded an excellent amplification signal. However, complete inhibition of PCR was observed with cells that were chlorinated with 50- or 75-ppm chlorine at either 50^oC or 80^oC. In the samples treated with 25-ppm chlorine, only a small amount of PCR product was obtained in the 50^oC samples, and no

product was detected in the 80°C samples. Therefore, the treatment of cells with chlorine concentrations as low as 25-ppm prohibited the DNA-based detection of the organism.

TABLE 13. PCR Inhibition Generated by the Action of Chlorine on *B. anthracis* Cells

Chlorination Temperature and Time	0-ppm Chlorine	25-ppm Chlorine	50-ppm Chlorine	75-ppm Chlorine
50°C 5 minutes	++	trace	--	--
80°C 1 minute	++	--	--	--

4.2.3. Potential Interference of Chlorinated Cells on Lambda Bacteriophage PCR.

To determine whether chlorinated *B. anthracis* cells could affect PCR amplification of DNA, PCR was conducted with Lambda bacteriophage DNA as template in the presence of cells that had been exposed to chlorine. In this experiment, *B. anthracis* cells (10^5) washed once with de-ionized water were suspended in 10-µl de-ionized water, exposed to 0-, 25- or 50-ppm chlorine at 50°C for 5 minutes, and then dosed with sodium thiosulfate to neutralize chlorine. PCR was conducted with cell suspensions and Lambda bacteriophage DNA (0.05 or 0.005 ng) as target template to determine whether the chlorine-exposed cells affected PCR.

Results. Amplification of Lambda bacteriophage DNA was reduced when PCR was conducted in the presence of *B. anthracis* cells that were exposed to 25- or 50-ppm chlorine (TABLE 14). The effect of the chlorinated cells was minimal with the higher quantity of Lambda bacteriophage DNA template in the PCR mixture.

TABLE 14. PCR Amplification of Lambda Bacteriophage DNA in the Presence of Chlorinated *B. anthracis* Cells

Chlorine (ppm)	0.05-ng Lambda DNA	0.005-ng Lambda DNA
0	+++	+++
25	++	++ (25% inhibition)
50	++	<+ (80% inhibition)

5. CHLORINE REACTIONS AS POTENTIAL SOURCES OF PCR INTERFERENCE.

Experiments were conducted to identify sources of PCR inhibition that may arise during the chlorination of *B. anthracis* spores or cells.

5.1. External Factors and Chlorine Reactivity as Potential Sources of PCR Interference.

5.1.1. Chlorine Reactivity with Standard Eppendorf Tube.

While a majority of the chlorination studies were performed using spores which had been heated with chlorine in Perkin Elmer (PE) brand PCR tubes, a few of the chlorination studies were performed using standard Eppendorf tubes. Greater levels of PCR interference were observed when the chlorine treatment of spores was performed in the standard PCR tubes. This experiment was performed to determine whether materials interfering with PCR were being generated during the chlorination in standard Eppendorf tubes as compared with the PE PCR tubes. The *capB* amplification product (0.2 µl per 10 µl reaction), which had been produced in a previous PCR run, was heated at either 80°C for 1 minute or 50°C for 5 minutes in the presence and absence of 25-ppm chlorine.

Results. In the absence of chlorine, PCR interference was not observed with either the Perkin Elmer (PE) brand PCR tubes or the standard Eppendorf tubes during heating (TABLE 15). There was also no inhibition of PCR when the chlorination reaction was performed in PE PCR tubes. Interestingly, the chlorination of *B. anthracis* template DNA in standard Eppendorf tubes at either 50°C or 80°C caused inhibition of PCR amplification. The PCR inhibition was more pronounced when the tubes were heating at 80°C. All further chlorination treatment reactions will be performed in the PE PCR tubes.

TABLE 15. PCR Amplification of *B. anthracis* Template DNA which was Chlorinated in either Standard Eppendorf Tubes or PE PCR Tubes at 50°C and 80°C

Temperature	Std. Ep. Tube 0-ppm Chlorine	PE Tube 0-ppm Chlorine	Std. Ep. Tube 25-ppm Chlorine	PE Tube 25-ppm Chlorine
50°C	++	++	+	++
80°C	++	++	trace	++

5.1.2. Influence of Water Source Quality on PCR.

To determine if water quality could influence the degree of PCR interference that could arise during chlorine exposure trials, the stability of DNA suspended in a given water source was evaluated on the basis of PCR-product yield. In this experiment, Lambda bacteriophage DNA was suspended in HPLC-grade water (two sources) or de-ionized water (dH_20), incubated at either 25°C or at 50°C, and then assayed by PCR. The evaluation was also conducted with a lower DNA concentration (40 ng/ml) with heating a 50°C.

Results. Each DNA sample that was suspended in HPLC-grade water amplified well (TABLE 16). About a 25% reduction in PCR signal was observed with the lower DNA concentration (40 ng/ml) suspended in dH_20 and heated at 50^oC. Perhaps impurities of the dH_20 interacted with the DNA affected quality of the template for PCR. Consequently, HPLC-grade water was used for the chlorination experiments.

TABLE 16. PCR of Lambda Bacteriophage DNA with Various Water Sources

Temperature (^oC)	Lambda DNA (ng/ml)	HPLC Water #1	HPLC Water #2	dH2O
25	80	+++	+++	+++
50	80	+++	+++	+++
50	40	+++	+++	++

5.1.3. Evaluation of *B. anthracis* PCR Primers (Operon Company) with Chlorinated and Unchlorinated Spores.

The use of new preparations of *B. anthracis* primers produced by the Operon company were evaluated with chlorinated samples. The use of 0.2- and 0.5-μM baF and baR primers for the PCR amplification of spores treated with 25-ppm chlorine at 50^oC for 5 minutes was compared.

Results. Good amplification of 10^6 non-chlorinated spores occurred when either 0.2 or 0.5 μM baF and baR primers were used (TABLE 17). There was no amplification of DNA in the spore sample chlorinated with 25 ppm. When 0.5 μM ba primers were used, there was a larger quantity of primer dimer formation. Therefore, the use of 0.2 μM ba primers should be sufficient to obtain good PCR results for the amplification of the *capB* gene.

TABLE 17. Use of New Primer Preparation for the PCR Amplification of Chlorinated and Non-Chlorinated *B. anthracis* Spores

Primer Concentration	0-ppm Chlorine	25-ppm Chlorine
0.2 μM	++	--
0.5 μM	++	--

5.2 Chlorine Reactivity with the *B. anthracis* Spore as a Potential Source of PCR Interference.

5.2.1. Effect of Spores Exposed to Elevated Temperatures in the Absence of Chlorine on Lambda Bacteriophage PCR.

To determine whether *B. anthracis* spores exposed to elevated temperature can release material that affects PCR, Lambda bacteriophage DNA (0.1 μg/ ml per reaction) was incubated with 10^3, 10^4, or 10^5 spores at ambient temperature or 80°C for 1 minute. The control consisted of Lambda bacteriophage DNA heated in the absence of spores.

Results. No PCR interference resulted from the incubation of 0.1 μg/ml Lambda bacteriophage DNA in the presence of anthrax spores at ambient temperature or 80°C (TABLE 18).

TABLE 18. PCR of Lambda Bacteriophage DNA following Incubation with *B. anthracis* Spores at 50°C and 80°C

Temperature	10^5 Spores	10^4 Spores	10^3 Spores	No Spores
50°C	++	++	++	++
80°C	++	++	++	++

5.2.2. PCR of Fresh and Aged Spores Exposed to Chlorine at 50°C for 5 Minutes.

In this experiment, PCR-based detection of spore preparations that had aged either 8 weeks (old spores) or 1 week (new spores) was compared. *B. anthracis* spores (7×10^4 per PCR mixture) were incubated for 5 minutes at ambient temperature or 50°C in the absence or presence of 25-ppm chlorine.

Results. Good PCR amplification occurred in all samples. However, there was a slight reduction (10 or 25%) in the signal strength for the chlorinated samples. The age of the spores had no apparent effect on the PCR amplification of chlorinated and unchlorinated spores (TABLE 19).

Table 19. PCR Amplification of Chlorinated and Unchlorinated *B. anthracis* Spores of 1 or 8 Weeks Age

Spore Age	Ambient temperature, 0-ppm Chlorine	50°C, 0-ppm Chlorine	50°C, 25-ppm Chlorine
1 week	++	++	++ (<10%)
8 weeks	++	++	++ (<25%)

5.2.3. PCR of Lambda Bacteriophage Genomic DNA in the Presence and Absence of Spores Exposed to Chlorine at 50°C for 5 Minutes.

Previous experiments revealed that the effects of PCR inhibitors were much more pronounced when the amplification was performed using low DNA template concentrations. In this experiment, the effect that chlorinated and non-chlorinated spores could have on the amplification of low concentrations of Lambda bacteriophage genomic DNA template was examined.

The PCR amplification of 0.005, 0.05, and 0.5-ng of Lambda bacteriophage DNA was performed in the presence of 10^5 spores which were either unchlorinated or had been chlorinated with 25-ppm chlorine at 50°C for 5 minutes. The chlorine treatment of spores was performed at 50°C for 5 minutes. The non-chlorinated spores were also incubated at 50°C for the same period of time. Sodium thiosulfate (10 mM) was added to all spore samples following the heat incubation step to neutralize the chlorine.

Results. PCR amplification of Lambda bacteriophage genomic DNA was not inhibited in the presence of 10^5 unchlorinated spores that had been heated at 50°C. However, PCR inhibition occurred in the presence of spores that had been treated with 25-ppm chlorine (TABLE 20).

TABLE 20. PCR of Lambda Bacteriophage DNA in the Presence of Chlorinated *B. anthracis* Spores

Lambda DNA (ng)	25-ppm Chlorine, 10^5 Spores	No Chlorine, 10^5 Spores	Controls (No Spores, No Chlorine)
0.5	+	+++	+++
0.05	<+	+++	++
0.005	<+	++	+

5.2.4. PCR Interference from Material Associated with Chlorinated Spores and Not from Material Released from Spores.

In this experiment, amplification of 0.005-, 0.05- and 0.5-ng Lambda bacteriophage genomic DNA was performed in the presence of:

a) spores exposed to 25-ppm chlorine at 50°C for 5 minutes,
b) spore-free filtrate (microcentrifugation filter tube with 0.22-μm pore diameter) of spores exposed to 25-ppm chlorine at 50°C for 5 minutes,
c) spore-free filtrate (microcentrifugation filter tube with 0.22-μm pore diameter) exposed to 25-ppm chlorine at 50oC for 5 minutes,
d) controls (no spores, no filtrate)

Results. PCR of Lambda bacteriophage genomic DNA was inhibited by approximately 50% in the presence of the chlorinated spores (TABLE 21). The spore-free filtrate of chlorinated spores was not inhibitory to PCR. These results suggest that the PCR inhibitors were retained with the spores and not being released into the filtrate wash.

TABLE 21. PCR in the Presence of Chlorinated Spores and the Spore-free Supernatant of Chlorinated *B. anthracis* Spores

Lambda DNA (ng)	Chlorinated Spores	Filtrate of Chlorinated Spores	Chlorinated Filtrate	Controls
0.5	++	+++	+++	+++
0.05	++	+++	+++	+++
0.005	++	+++	+++	+++

5.2.5. Interference of Lambda Bacteriophage PCR by Fractions Spores Exposed to Chlorine at 50°C for 5 Minutes.

To confirm that the PCR inhibitors generated during chlorination remain with the spores, Lambda bacteriophage genomic DNA (0.5 ng) was added to 10 μl of the following suspensions or supernatant:

a) non-chlorinated spores (1.5×10^6),
b) spores (1.5×10^6) treated with 75-ppm chlorine then neutralized with 10-mM sodium thiosulfate,
c) spore-free supernatant of spores treated with 75-ppm chlorine obtained by microfiltration of the chlorinated spore suspension through a 0.22-μm microcentrifugation filter,
d) chlorinated spores which had been retained by the microfiltration apparatus and resuspended in 100-μl deionized water (10 μl of the suspension was used for PCR),
e) control (no spores or supernatant).

Results. PCR of Lambda bacteriophage DNA was not inhibited in the presence of non-chlorinated spores (TABLE 22). In addition, PCR was not inhibited by the spore-free filtrate that was obtained following chlorination. However, the chlorinated spores, as well as the chlorinated spores that were retained after microfiltration, were inhibitory to PCR.

TABLE 22. PCR of Lambda Bacteriophage DNA following Incubation with Chlorinated *B. anthracis* Spores and Spore-free Extracts

Unchlorinated Spores, 50°C	Chlorinated Spores, 50°C	Fraction #1: Spore-free Extract of Chlorinated Spores	Fraction #2: Chlorinated Spores Retained during Microfiltration	Lambda Control DNA
+++	++	+++	++	+++

5.2.6. Possible Binding Interactions between Chlorinated Spores and PCR Components.

To determine whether there is a non-specific binding of primers to chlorinated spores, the PCR master mix (lacking *Taq* DNA polymerase and Lambda bacteriophage DNA template) was incubated in the presence of unchlorinated spores as well as spores that had been chlorinated with 75-ppm chlorine at 50°C for 5 minutes. The chlorinated spore suspension was then neutralized with 10-mM sodium thiosulfate and the spores were centrifuged with a 0.22-μm microfiltration tube. The master mix was added to the spores retained by the microcentrifugation filter, vortexed, then centrifuged to collect the PCR master mix that had been incubated with the chlorinated spores. The master mix was then used for the amplification of Lambda bacteriophage DNA (*Taq* DNA polymerase and DNA were added following microfiltration).

Results. Good amplification was obtained in all filtered samples (TABLE 23). Incubation of primers in the presence of chlorinated spores had no detrimental effect on the amplification of DNA. Thus, there was no apparent binding of the primers to the chlorinated spores. These results suggest that PCR interfering material was associated with the spores and was not present in the filtrate of the chlorinated spores.

TABLE 23. PCR Amplification of Lambda Bacteriophage DNA following Incubation of PCR Master Mix in the Presence or Absence of Chlorinated *B. anthracis* Spores

MICROFILTRATION CONDITIONS	AMPLIFICATION PRODUCT
Master Mix Filtered through Unchlorinated Spores	+++
Master Mix Filtered through Chlorinated Spores	+++
Master Mix Filtered in the Absence of Spores	+++

6. PCR OF DNA EXPOSED TO CHLORINE.

6.1 Chlorination of Lambda Bacteriophage DNA.

Although the PCR master mix does not consume a sizable amount of chlorine, it is possible that the buffering action of the PCR mix helps stabilize the DNA during chlorination. In the spore chlorination experiments, the spores were suspended in deionized water during the

chlorination. Therefore, the chlorination of DNA should also be performed in deionized water. The following chlorination experiments were performed using Lambda bacteriophage genomic DNA or purified PCR amplification product. The PCR product was purified using the GeneClean kit (Bio-101 Company). The DNA preparations were suspended in deionized water.

6.1.1. Effect of Exposure to 0-, 25-, 50-, or 75-ppm Chlorine on PCR of Lambda Bacteriophage DNA.

Lambda bacteriophage genomic DNA (Perkin Elmer stock solution, 1-µg DNA/ml) was treated with either 0-, 25-, 50- or 75-ppm chlorine for 1 minute at 85^{o}C. For each reaction, 0.1-ng DNA was chlorinated. The chlorine was neutralized with 10-mM sodium thiosulfate and the samples were used directly for PCR.

Results. Genomic DNA which was chlorinated with either 25-, 50- or 75-ppm chlorine could not be amplified using PCR. In the absence of chlorine, the DNA template was successfully amplified by PCR (TABLE 24).

TABLE 24. PCR Amplification of Lambda Bacteriophage Genomic DNA Treated with 0-, 25-, 50-, or 75-ppm Chlorine at 85^{o}C for 1 Minute

0-ppm Chlorine	25-ppm Chlorine	50-ppm Chlorine	75-ppm Chlorine
+++	--	--	--

6.1.2. Effect of Exposure to 50- or 400-ppm Chlorine on PCR and Structural Integrity of Lambda Bacteriophage DNA (PCR Product).

The following series of experiments were performed to determine whether the modification or degradation of the Lambda bacteriophage DNA (PCR product) by chlorine could be detected by agarose gel electrophoresis. For these experiments, the chlorination reactions were performed using quantities of purified Lambda bacteriophage PCR product that could be visualized by agarose gel electrophoresis and ethidium bromide staining. Since the lower limit of detection using ethidium bromide on a agarose gel is 0.01-µg DNA, sufficient quantities of DNA were used to obtain a sizeable band on an agarose gel. If the chlorinated DNA template appeared altered as visualized on an agarose gel, then PCR was performed to determine whether the remaining material could be amplified by PCR.

In this experiment, 0.36-µg amounts of Lambda bacteriophage DNA (PCR product) as 10-µl volumes in PCR reaction tubes were chlorinated with either 50- or 400-ppm chlorine. The samples were heated at 85^{o}C for 1 minute and then received additional heating in the Perkin Elmer thermal cyler (Model 9600) using PCR program 16. After the thermal cycling, 8-µl of each sample was fractionated through 1% agarose and stained with ethidium bromide to visualize the condition of the DNA. If DNA appeared damaged as indicated by band smearing

or reduction in fluorescence intensity, 1 μl of the remaining DNA suspension was assayed by PCR to determine whether the template could be amplified by PCR.

Results. The Lambda bacteriophage DNA that was treated with 50-ppm chlorine appeared largely intact, whereas the DNA treated with 400-ppm chlorine appeared to have undergone partial degradation. Degradation was indicated by smearing and reduced intensity of the DNA band as visualized by gel electrophoresis and ethidium bromide staining (TABLE 25). Degradation of the template during exposure to 400-ppm chlorine was further confirmed when the material failed to serve as a template for PCR (Table 26).

TABLE 25. Visual Appearance of Lambda Bacteriophage DNA (PCR Product) after Chlorine Exposure at 85°C for 1 minute and Thermal Cycler Temperatures

	50-ppm Chlorine	400-ppm Chlorine
DNA Degradation	None	Partial

Table 26. PCR Amplification of Lambda Bacteriophage DNA (PCR Product) after Chlorine Exposure at 85°C for 1 minute and Thermal Cycler Temperatures

PCR-tested DNA	50-ppm Chlorine	400-ppm Chlorine
Undiluted (36 ng)	+++	--
Diluted 0.1x (3.6 ng)	++	--

6.1.3. Effect of Exposure to 50-, 100-, or 400-ppm Chlorine on PCR and Structural Integrity of Lambda Bacteriophage DNA (PCR Product).

This experiment was performed to confirm the degradation of the Lambda bacteriophage DNA by exposure to chlorine as observed in the above section. The previoius experiment was repeated using a wider range of chlorine concentrations for exposure.

Results. The chlorination of the Lambda amplification product with 400-ppm chlorine caused degradation of the template. In addition, no PCR amplification product was generated using this template DNA. At chlorine concentrations of 100 ppm or lower, the template appeared intact. In the 50-ppm sample, there was a actually a weaker band than in the 100 ppm sample. The PCR amplification of the DNA treated with 50 ppm chlorine yielded less amplification product than the 100 ppm sample (Tables 27 and 28). It is possible that there was less DNA present during the chlorination reaction than was intended.

Table 27. Appearance of Lambda Bacteriophage DNA (PCR Product) after Chlorination at 85°C for 1 Minute and Thermal Cycling Temperatures

	0-ppm Chlorine	50-ppm Chlorine	100-ppm Chlorine	400-ppm Chlorine
DNA Degradation	None	None	None	Extensive

Table 28. PCR Amplification Using Lambda Bacteriophage DNA (PCR Product) Chlorinated with 0-, 50-, 100-, and 400-ppm Chlorine at 85°C and Thermal Cycler Temperatures

PCR-tested DNA	0-ppm Chlorine	50-ppm Chlorine	100-ppm Chlorine	400-ppm Chlorine
Undiluted (36 ng)	+++	+ (weak)	++	--
Diluted 0.1x (3.6 ng)	++	+ (weak)	++	--

6.1.4. Effect of Exposure to 0-, 50-, 100-, 200-, or 400-ppm on PCR and Structural Integrity of Lambda Bacteriophage DNA (PCR Product).

In this experiment, DNA was evaluated using a smaller quantity (0.25 μg) than was employed in the above experiment (0.36 μg). For these trials, 0.25-μg amounts of Lambda bacteriophage amplification product were chlorinated with either 0-, 50-, 100-, 200-, 400-, or 500-ppm chlorine. The samples were heated at 85°C for 1 minute, neutralized with 10-mM sodium thiosulfate, and then heated in the Perkin Elmer thermal cyler (Model 9600) using PCR program 16. The total reaction volume was 10 μl. After the thermal cycling, 8-μl of each sample was fractionated through 1% agarose and stained with ethidium bromide to assess the visual integrity of the template. DNA degradation was indicated by smearing and reduced intensity of bands. In addition, 1 μl of each chlorinated DNA suspension was evaluated by PCR to determine whether the chlorinated template could be amplified.

Results. The DNA was degraded by the chlorine in the samples exposed to 200- or greater ppm chlorine (TABLES 29 and 30). For these samples, target DNA was not amplified using PCR. DNA treated with 0-, 50-, or 100-ppm chlorine did not appear damaged by visual inspection of the agarose gel and was suitable for amplification by PCR.

Table 29. Appearance of Lambda Bacteriophage DNA (PCR Product) after Chlorination at 85°C for 1 Minute and Thermal Cycling Temperatures

	0-ppm Chlorine	5-ppm Chlorine	100-ppm Chlorine	200-ppm Chlorine	400-ppm Chlorine	500-ppm Chlorine
DNA Degradation	None	None	None	Partial	Extensive	Extensive

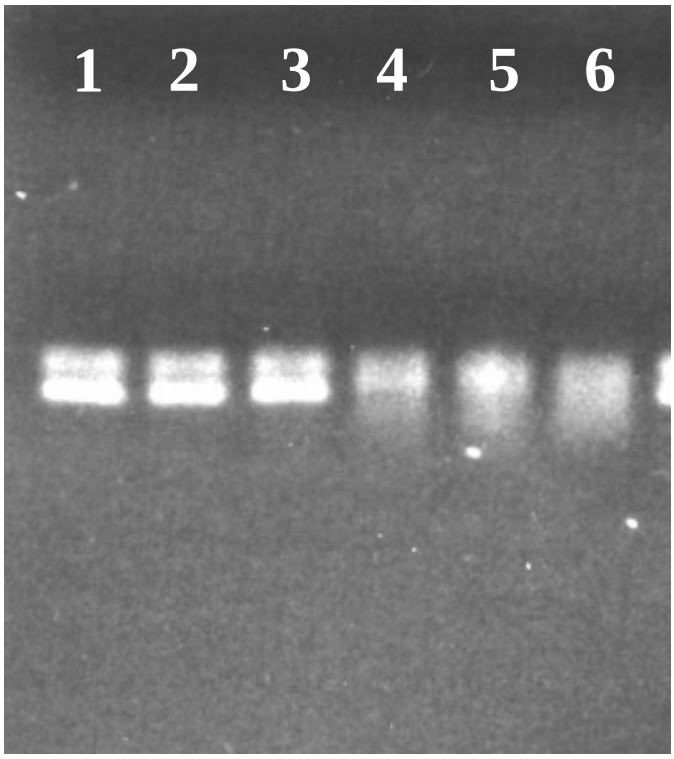

FIGURE 2. Effects of Chlorine Exposure (85°C for 1 Minute and Thermal Cyling Temperatures) on Lambda Bacteriophage DNA (PCR Product) as Observed by Agarose Gel Electrophoresis. Lane number (chlorine concentration): 1 (0 ppm), 2 (5 ppm), 3 (100 ppm), 4 (200 ppm), 5 (400 ppm), 6 (500 ppm).

TABLE 30. PCR Amplification of Lambda Bacteriophage DNA (PCR Product) after Chlorine Exposure at 85°C for 1 minute and Thermal Cycler Temperatures

PCR-tested DNA	0-ppm Chlorine	50-ppm Chlorine	100-ppm Chlorine	200-ppm Chlorine	400-ppm Chlorine	500-ppm Chlorine
25 ng	+++	+++	+++	--	--	--

6.2. Chlorine Reactivity with *B. anthracis* DNA.

The following experiments were performed to evaluate degradation of *B. anthracis* DNA by chlorine as determined by agarose gel electrophoresis. For these experiments, the chlorination reactions were performed using purified *B. anthracis* DNA amplification product. The PCR target was a 623-bp sequence of the *CapB* region of pX02. If a chlorinated DNA sample appeared altered as indicated by agarose gel electrophoresis, PCR was performed with the sample to determine whether the target was capable of being amplified by PCR.

B. anthracis amplification product was generated by conducting PCR under standard conditions using PCR program 16. Amplification product was purified using the Geneclean kit (BIO-101). For these trials, samples which contained 0.32-µg of *B. anthracis* DNA were exposed to 0-, 50-, 100-, 200-, 400- or 500-ppm chlorine. The samples were heated at 85^{o}C for 1 minute, neutralized with an excess of sodium thiosulfate, and then heated in the Perkin Elmer thermal cyler (Model 9600) using PCR program 16. In addition, a second set of chlorination reactions was performed in the presence of spores (1.7 x 10^{5}).

Results. The effects of chlorine on the *B. anthracis* PCR product were similar to those observed with chlorinated Lambda bacteriophage PCR product. Exposure to a chlorine concentration of 200 ppm or greater appeared to partially degrade the DNA as visualized with agarose gel electrophoresis (FIGURE 3 and TABLE 31). When PCR was performed with these samples, quantities of PCR amplification product generated were reduced (TABLE 32). For samples that exhibited altered PCR product as visualized by gel electrophoresis, less PCR product was generated. The DNA template that was treated with 0-, 50- or 100-ppm chlorine did not appear to be altered as indicated by gel electrophoresis. In addition, these samples generated PCR product.

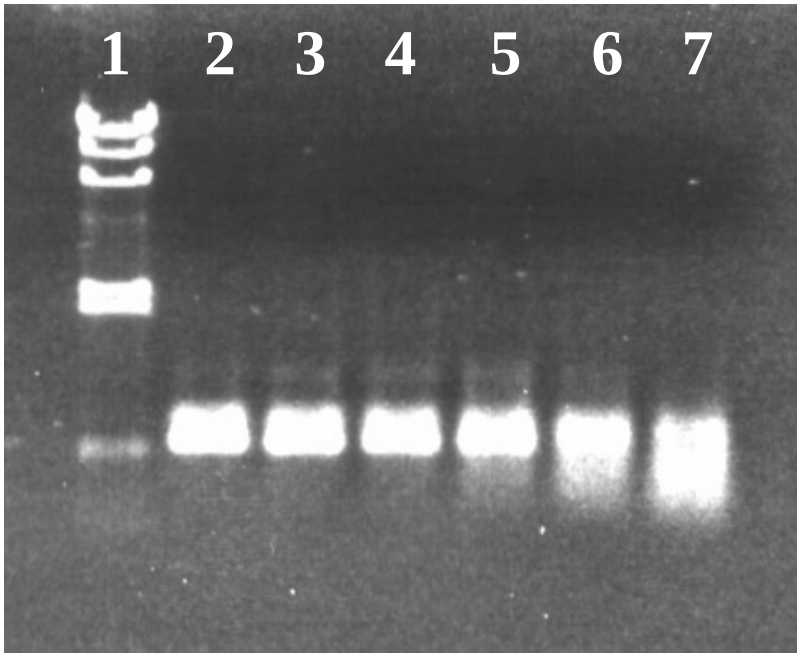

FIGURE 3. Effects of Chlorine Exposure (85^{o}C for 1 Minute and Thermal Cyling Temperatures) on *B. anthracis* DNA (PCR Product) as Observed by Agarose Gel Electrophoresis. Lane number (chlorine concentration): 2 (0 ppm), 3 (50 ppm), 4 (100 ppm), 5 (200 ppm), 6 (400 ppm), 7 (500 ppm). Lane 1, bp length of DNA fragments (*Hin*dIII-digest of Lambda bacteriophage DNA) from top to bottom: 23,130; 9,416; 6,557; 4,361; 2,322; 2,027; and 564.

TABLE 31. Appearance of *B. anthracis* DNA (PCR Product) after Chlorination at 85°C for 1 Minute and Thermal Cycling Temperatures

	0-ppm Chlorine	50-ppm Chlorine	100-ppm Chlorine	200-ppm Chlorine	400-ppm Chlorine	500-ppm Chlorine
DNA Degradation	None	None	None	Partial	Partial	Partial

TABLE 32. PCR Amplification of *B. anthracis* DNA (PCR Product) after Chlorine Exposure at 85°C for 1 Minute and Thermal Cycler Temperatures

PCR-test DNA	0-ppm Chlorine	50-ppm Chlorine	100-ppm Chlorine	200-ppm Chlorine	400-ppm Chlorine	500-ppm Chlorine
32 ng	+++	+++	+++	++	+ (weak)	--

6.3 Impact of Chlorine-to-DNA Ratio on Integrity of Lambda Bacteriophage DNA.

This experiment was conducted to determine the relationship between chlorine dose and DNA quantity as it relates to the degree of chlorine-mediated alteration or degradation of Lambda bacteriophage DNA. Various quantities (0.04, 0.2, and 0.4 μg) of Lambda bacteriophage genomic DNA (*Hin*dIII digest, high molecular weight ladder, BioRad) were exposed to 0-, 25-, 50-, 100-, 150-, 200-, or 400-ppm chlorine in a 10-μl total volume at 85°C for 1 minute. Immediately following chlorine exposure, the samples were fractionated by gel electrophoresis. Gels were stained with ethidium bromide to visualize any DNA alterations.

Results. To understand how chlorine exposure could alter DNA, findings were evaluated to determine how the ratio of chlorine molecules to DNA molecules impacted DNA integrity (FIGURE 4, TABLES 33 and 34). Considering (1) Lambda bacteriophage genomic DNA consists of 48,502 bp and (2) one bp has a molecular weight of 635 Daltons, a quantity of 0.4-μg DNA would consist of 3.8×10^{14} bp. Thus, for the highest chlorine concentration (100 ppm) that did not affect DNA integrity, the chlorine molecule-to-bp ratio was 4.5. For the lowest chlorine concentration (150 ppm) that exerted an effect on the DNA, the chlorine-to-bp ratio was 67.0. Thus, a great excess of chlorine may be needed to degrade DNA.

The need for high chlorine excess for degradation of Lambda bacteriophage DNA was also observed with a lower quantity of DNA. For reactions with 0.2-μg DNA, no effect on DNA integrity was observed with exposure to 50-ppm chlorine which provided a chlorine-to-bp ratio of 44.7. For the lowest chlorine concentration (100 ppm) that affected integrity of 0.2-μg DNA, the chlorine-to-bp ratio was 89.4.

Chlorine exposure appeared to exert two types of effects on Lambda bacteriophage DNA as observed by gel electrophoresis. First, lower chlorine doses altered electrophoretic mobility by increasing the apparent size of some of the *Hin*dIII-digest fragment species. This was noted with 0.4-μg DNA exposed to 150- or 200-ppm chlorine and 0.2-μg DNA exposed to 100-ppm chlorine. This could be the result of substitution reactions that add chlorine atoms to nucleotide

bases. Such additions could increase the mass of DNA fragments and alter electrophoretic mobility. Second, exposure to higher chlorine doses rendered material that did not stain with ethidium bromide. This was observed with 0.4-μg DNA exposed to 400-ppm chlorine, 0.2-μg DNA exposed to 200-ppm chlorine, and 0.04-μg DNA exposed to 25-ppm chlorine. Perhaps the elevated chlorine doses denatured or degraded the DNA so that there was insufficient double-stranded structure required for interchelation of the dye.

The ratio of chlorine molecules to base pairs of Lambda bacteriophage DNA appeared to reflect the effect of the halogen on the DNA as observed by gel electrophoresis. No effect was evident with ratios of 44.7, 4.5, or lower. Increased apparent molecular weight was observed with ratios of 67.0 and 89.4. Significant degradation as indicated by no visible material following ethidium bromide staining was observed with ratios of 111.7, 134.1, and 1,116.8. Thus, purified DNA appears to maintain basic structural integrity upon exposure to high doses of chlorine.

FIGURE 4. Effects of Chlorine Exposure at 85°C for 1 Minute on Lambda Bacteriophage DNA (*Hind*III Digest) as Observed by Agarose Gel Electrophoresis.
0.4–μg DNA, lane number (chlorine concentration): 1 (0 ppm), 2 (25 ppm) 3 (50 ppm), 4 (100 ppm), 5 (150 ppm), 6 (200 ppm), 7 (400 ppm)
0.2-μg DNA, lane number (chlorine concentration): 8 (0 ppm), 9 (25 ppm) 10 (50 ppm), 11 (100 ppm), 12 (150 ppm), 13 (200 ppm), 14 (400 ppm)
0.04-μg DNA, lane number (chlorine concentration): 15 (0 ppm), 16 (25 ppm) 17 (50 ppm), 18 (100 ppm), 19 (150 ppm), 20 (200 ppm), 21 (400 ppm)

TABLE 33. Effects of Chlorine Exposure at 85°C for 1 Minute on Lambda Bacteriophage DNA (*Hin*dIII Digest) as Observed by Agarose Gel Electrophoresis

DNA Amount	0 ppm Chlorine	25 ppm Chlorine	50 ppm Chlorine	100 ppm Chlorine	150 ppm Chlorine	200 ppm Chlorine	400 ppm Chlorine
0.4 μg	++	++	++	++	+*	+*	--
0.2 μg	++	++	++	+*	--	--	--
0.04 μg	++	--	--	--	--	--	--

++ = No effect
+* = Increase in apparent molecular weight of the DNA bands
-- = No visible material

TABLE 34. Effect of Chlorine-to-Base Pair Ratio on Integrity of Lambda Bacteriophage DNA (*Hin*dIII Digest) Exposed to Chlorine at 85°C as Observed by Agarose Gel Electrophoresis

Chlorine-to-DNA Base Pair Ratio	No Observed Effect	Increase in Apparent MW of DNA	No Visible Material
≤ 4.5	+		
44.7	+		
67.0		+	
89.4		+	
111.7			+
134.1			+
≥ 1,116.8			+

The relationship between chlorine molecule number and the denaturation or degradation of DNA was determined by examining DNA following agarose gel electrophoresis and ethidium bromide staining. The absence of ethidium bromide-stainable DNA following chlorine treatment could be attributed to denaturation of double stranded DNA or degradation of the molecule. The relationship is displayed in Figure 5. Linear regression analysis indicated a positive correlation between the chlorine dose and alteration of DNA ($r^2 = 0.98$). Based on this analysis, 194 nmoles of chlorine is capable of altering 1.0 μg of double stranded DNA so that the material is not visible following ethidium-bromide staining. This translates to a chlorine-to-base pair ratio of 123.

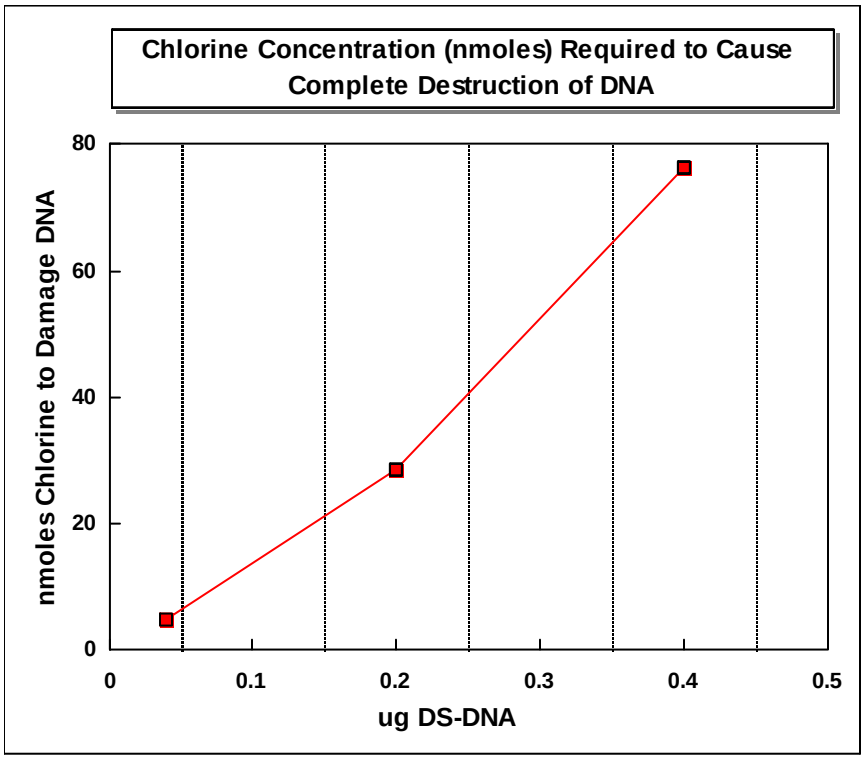

FIGURE 5. Relationship between Quantities of Chlorine and Lambda Bacteriophage DNA (*Hin*dIII Digest) with DNA Integrity as Observed by Agarose Gel Electrophoresis.

6.4. Impact of Chlorine-to-DNA Ratio on Integrity of Lambda Bacteriophage DNA (PCR Product).

This section includes trials that were conducted using PCR product of Lambda bacteriophage genomic DNA to investigate the effect of chlorine on (1) PCR template and (2) integrity of the DNA structure as determined by gel electrophoresis. For these experiments, the ratio of chlorine molecules to base pairs was considered in order to understand effects of the halogen exposure on the DNA. Based on (1) one base pair having a molecular weight of 635 Daltons, (2) one µl of PCR product containing 0.1-µg DNA (by comparison with molecular weight standard DNA) and, thus, 9.5 x 10^{13} bp, and (3) 500 bp per Lambda bacteriophage PCR product.

6.4.1. Lack of Chlorine Effect on Lambda Bacteriophage DNA at Low Chlorine-to-DNA Ratio.

In this experiment, Lambda bacteriophage PCR product was exposed to 0-, 10- or 25-ppm chlorine in a 10-µl volume for 1 minute at 85^{o}C. Following exposure, chlorine was neutralized with 10-mM sodium thiosulfate. Treated DNA was employed as template for PCR using thermal cyling method 16 as described in an above section. Suitability of the DNA as PCR

template was determined by fractionating the product through 1% agarose and staining with ethidium bromide.

Results. Compared with the control without chlorine, exposures to the halogen had no apparent effect on the DNA with respect to effectiveness as PCR template (TABLE 35). This could be attributed to the low ratio of chlorine molecules to DNA base pairs. For the higher chlorine level (25 ppm), there were only 4.5 chlorine molecules per bp. This ratio could be insufficient for affecting the DNA as PCR template.

TABLE 35. Effect of Chlorine Exposure at 85°C for 1 Minute on Lambda Bacteriophage DNA (PCR Product) as PCR Template

Chlorine (ppm)	Strength of PCR Signal
0	++
10	++
25	++

6.4.2. Effect of Chlorine on Lambda Bacteriophage DNA (PCR Product) at High Chlorine-to-DNA Ratio.

This experiment was performed to investigate the impact of the ratio of chlorine to DNA on DNA integrity. Various amounts of Lambda bacteriophage PCR product were exposed to 0- and 50-ppm chlorine at 85°C for 1 minute. Following exposure, chlorine was neutralized with 10-mM sodium thiosulfate. The samples were fractionated by agarose gel electrophoresis and stained with ethidium bromide to observe DNA.

Results. Compared with controls, chlorine exposure of the 5- or 10-µl volumes of PCR product did not significantly alter DNA as observed by gel electrophoresis and ethidium bromide staining (TABLE 36). However, no DNA was observed with 2-µl PCR product exposed to chlorine. These data suggest that a chlorine molecule-to-bp ratio of 44.8 (for the 2-ul sample) was sufficient to alter the DNA. The lower ratios (17.9 for the 5-µl sample and 9.0 for the 10-µl sample) may not have been adequate to alter the DNA.

TABLE 36. Effect of Chlorine Exposure at 85°C for 1 Minute on DNA Integrity for Various Amounts of Lambda Bacteriophage DNA (PCR Product)

	DNA Band Intensity		
Chlorine Concentration	**10-µl PCR Product**	**5-µl PCR Product**	**2-µl PCR Product**
0 ppm	+++	++	+
50 ppm	+++	++	--

6.5. Influence of *B. anthracis* Spores on the Effect of Chlorine on the Integrity Lambda Bacteriophage DNA.

Experiments presented above indicated that chlorine exposure at elevated temperatures altered genomic DNA or PCR product. However, chlorine exposure in the presence of *B. anthracis* spores could influence the effect of the halogen on DNA. Such an effect would be significant since chlorine treatment of *B. anthracis* would most likely involve exposure of both the spore and its DNA to chlorine.

6.5.1. Lambda Bacteriophage DNA (PCR Product) Exposed to Chlorine in the Presence of *B. anthracis* Spores at 85°C.

This experiment was conducted to determine if the presence of *B. anthracis* spores during chlorine treatment could impose an effect on DNA integrity. Various amounts of Lambda bacteriophage PCR product were exposed to 0- and 50-ppm chlorine at 85°C for 1 minute with or without dormant *B. anthracis* spores (about 2 x 10^5). Following exposure, chlorine was neutralized with 10-mM sodium thiosulfate. The samples were fractionated by agarose gel electrophoresis and stained with ethidium bromide to observe DNA.

Results. The presence of *B. anthracis* spores appeared not to influence the effect of chlorine exposure on the integrity of the DNA (TABLE 37). As in the above experiment (TABLE 36), the exposure with the highest chlorine-to-DNA ratio (44.8 for the 2-µl sample) altered the DNA as indicated by no observable DNA band. For each sample with the lower chlorine-to-DNA ratios (17.9 for the 5-µl sample and 9.0 for the 10-µl sample), chlorine exposure had no effect on DNA.

TABLE 37. Effect of Chlorine Exposure at 85°C for 1 Minute on DNA Integrity of Lambda Bacteriophage DNA (PCR Product) in the Presence of *B. anthracis* Spores

	DNA Band Intensity		
Chlorine Concentration	**10-µl PCR Product**	**5-µl PCR Product**	**2-µl PCR Product**
0 ppm	+++	++	+
0 ppm + Spores	+++	++	+
50 ppm	+++	++	--
50 ppm + Spores	+++	++	--

6.5.2. Lambda Bacteriophage DNA (PCR Product) Exposed to Chlorine in the Presence *B. anthracis* Spores at 85°C followed by Thermal Cycling Temperatures.

To follow trials conducted with *B. anthracis* spores exposed to chlorine with heat, Lambda bacteriophage DNA (PCR product) mixed with domant spores (about 2 x 10^5) were exposed to chlorine at 85°C for 1 minute and then heated with PCR thermal cycle method 16.

Following exposure, chlorine was neutralized with 10-mM sodium thiosulfate. The samples were fractionated by agarose gel electrophoresis and stained with ethidium bromide to observe DNA.

Results. As in the above experiment (TABLE 37), the presence of *B. anthracis* spores appeared not to influence the effect of chlorine exposure on the integrity of the DNA (TABLE 38). An effect on DNA integrity was only observed with the highest chlorine concentration (500 ppm). This was noted for samples with the higher chlorine-to-DNA ratios, being 179.1 for the 5-µl sample and 448.0 for the 2-µl sample. For these samples, no DNA band was evident. No effect on DNA integrity was observed for the 500-ppm exposure with the 10-µl sample, which had a chlorine-to-DNA ratio of 89.6.

TABLE 38. Effect of Chlorine Exposure at 85°C for 1 Minute (followed by Thermal Cycling Temperatures) on DNA Integrity of Lambda Bacteriophage DNA (PCR Product) in the Presence of *B. anthracis* Spores

	DNA Band Intensity		
Chlorine Concentration	**10-µl**	**5-µl**	**2-µl**
0 ppm	+++	++	+
50 ppm	**+++**	**++**	**+**
50 ppm + Spores	+++	++	+
500 ppm	+++	--	--
500 ppm + Spores	+++	--	--

6.6. Influence of Temperature on the Effect of Chlorine Exposure on Lambda Bacteriophage DNA.

Experiments presented above displayed effects of chlorine exposure on DNA with exposure to the halogen at an elevated temperature, such as 85°C. To determine if DNA alteration could occur at lower exposure temperatures, Lambda bacteriophage genomic DNA (0.1 µg) was exposed to a range of temperatures and chlorine concentrations for 1 minute. Following exposure, samples were neutralized with 10-mM sodium thiosulfate. DNA alteration was determined by assaying the samples with PCR using thermal cycling method 16.

Results. Only exposure to the highest temperature (85°C) altered effectiveness of the DNA as a template for PCR (TABLE 39). In addition, the effect was only observed with the highest chlorine concentration (50 ppm), which had a chlorine-to-bp ratio of 89.4. The next lower chlorine concentration (25 ppm), which yielded no effect on PCR, had a chlorine-to-bp ratio of 44.7.

TABLE 39. PCR Amplification of Lambda Bacteriophage Genomic DNA (0.1 μg/ml) Exposed to Various Temperatures and Chlorine Concentrations

Temperature	0-ppm Chlorine	10-ppm Chlorine	25-ppm Chlorine	50-ppm Chlorine
Ambient	++	++	++	++
37°C	++	++	++	++
50°C	++	++	++	++
85°C	++	++	++	--

7. EVALUATION OF METHODS TO REVERSE EFFECTS OF PCR INTERFERENCES CREATED BY CHLORINE EXPOSURE.

7.1 Addition of Bovine Serum Albumin (BSA).

7.1.1. PCR of Lambda Bacteriophage DNA in the Presence of Chlorinated BSA.

Since the PCR inhibition has occurred in the presence of chlorinated spores but not spore-free filtrate, the inhibitory substances appear to be retained with the spores. It is possible that chlorine exposure with heat is altering spore biomolecules such as proteins that can bind with PCR primers and template. In this experiment, the effects that heat-denatured and chlorinated BSA have on the PCR amplification of Lambda bacteriophage genomic DNA were examined. Sodium thiosulfate (10 mM) was added to all samples to neutralize free available chlorine prior to PCR. BSA (6.4 μg/ml) was either:

a) incubated at ambient temperature (no chlorine),
b) heated at 50°C for 5 minutes (no chlorine), or
c) heated at 50°C for 5 minutes in the presence of 25-ppm chlorine.

Results. There was no inhibition of Lambda bacteriophage DNA PCR in the presence of BSA that was chlorinated or heated (TABLE 40).

TABLE 40. PCR of Lambda Bacteriophage Genomic DNA Conducted in the Presence of Chlorinated and Heated BSA

BSA – 25°C 0-ppm Chlorine	BSA – 50°C 0-ppm Chlorine	BSA – 50°C 25-ppm Chlorine	Control (No BSA)
++	++	++	++

7.1.2. PCR of Lambda Bacteriophage DNA Exposed to Chlorine in the Presence of BSA.

Lambda bacteriophage genomic DNA was exposed to chlorine in the presence and absence of BSA at 25°C for 5 minutes or 85°C for 1 minute. This experiment was performed to determine whether BSA could protect DNA from the damaging effects of chlorine. During the chlorination reaction, the concentration of Lambda bacteriophage DNA was 1 ng/ml and the concentration of BSA was 1.6 μg/μl. The addition of BSA to PCR reaction mixtures has been reported to bind a variety of substances, which are known to be inhibitory to PCR. The optimal concentration of BSA for the relief of PCR inhibition from humic acids was reported to range from 200 to 400 ng/μl. This experiment was performed to determine whether the addition of BSA to the reaction mix could promote binding the PCR inhibitors that may be generated during chlorination

Results. There was uninhibited PCR of target DNA that was exposed to 25- or 75-ppm chlorine in the presence of BSA (TABLE 41). In the absence of BSA, there was no amplification of the chlorinated template DNA. It is possible that BSA protected DNA by consuming chlorine and reducing the residual.

TABLE 41. Chlorination of Lambda Bacteriophage Genomic DNA in the Presence and Absence of BSA at Ambient Temperature or 85°C

Chlorine (ppm)	Ambient Temperature (5 Minutes)		85oC (1 Minute)	
	No BSA	BSA (1.6 μg/ml)	No BSA	BSA (1.6 ug/ml)
0	+	+	+	+
25	-	+	-	+
75	-	+	-	+

7.1.3. PCR of *B. anthracis* Spores Exposed to Chlorine in the Presence of BSA.

Since uninhibited PCR amplification of chlorinated Lambda bacteriophage DNA was achieved in the presence of BSA (TABLE 41), the experiment was repeated in the spores. In this experiment, dormant *B. anthracis* spores (5×10^5 per reaction) were exposed to 0-, 25-, 50-, or 100-ppm chlorine in the presence or absence of BSA (1.6 μg/μl). The chlorination reaction was performed at 85°C for 1 minute and then the chlorine was neutralized with 10-mM sodium thiosulfate. PCR amplification of *B. anthracis* template using thermal cycling method 16 as described above was conducted.

Results. Uninhibited PCR amplification of spore target DNA occurred in the presence of chlorine when BSA was added to the reaction mixture (TABLE 42). The PCR signal was comparable to that of the control.

TABLE 42. PCR Amplification of *B. anthracis* Spores Chlorinated in the Presence of BSA

ppm Chlorine	No BSA	BSA (1.6 μg/ml)
0	+	+
25	-	+
50	-	+
100	-	+

7.1.4. Chlorine Consumption by BSA.

Consumption of chlorine by BSA was determined by amending chlorine solutions with the protein and then quantifying free chlorine residuals using the DPD (N,N-diethyl-p-phenylenediamine) colorimetric method.

Results. Chlorine solutions of a range of concentrations were greatly reduced by reaction with BSA (TABLE 43). Therefore, the PCR amplification of chlorinated spores that occurred in the presence of BSA (TABLE 42) could be attributed to reduction of the free available chlorine resulting from chlorine reactivity with BSA.

TABLE 43. Chlorine Consumption by BSA

Chlorine Dose (ppm)	BSA (μg/μl)	Chlorine after BSA (ppm)	Chlorine Consumed (Percent)
11.25	1.6	2.66	76.4
27	1.6	3.19	88.1
54	1.6	4.04	92.5
54	2.4	3.34	93.8

7.1.5. PCR of *B. anthracis* Spores Treated with BSA Immediately following Chlorine Exposure

Exposure of *B. anthracis* spores to chlorine in the presence of BSA produced material that did not adversely affect PCR (TABLE 42). However, as suggested by findings presented in section 7.1.4, the BSA may have consumed a great proportion of the chlorine and, thereby, yielded a low chlorine residual that had no effect on PCR. Thus, PCR amplification of chlorinated spores in the presence of BSA was similar to that of the non-chlorinated control spores. In this experiment, dormant *B. anthracis* spores (5×10^5 per reaction) were treated with BSA (1.6 μg/μl) immediately following exposure to 0-, 25-, 50-, or 100-ppm chlorine at 85°C for 1 minute and then BSA (1.6 μg/μl). The mixtures were then evaluated for the presence of material that could interfere with PCR. PCR amplification of *B. anthracis* target DNA was conducted using thermal cycling method 16.

Results. BSA added immediately following the chlorination reaction failed to negate PCR interference generated by the interaction of chlorine with dormant spores (TABLE 44).

TABLE 44. PCR Amplification of *B. anthracis* Spores Amended with BSA Immediately following Exposure to Chlorine

Chlorine (ppm)	No BSA	BSA (1.6 μg/μl)
0	++	++
25	-	-
50	-	-
100	-	-

7.1.6. Microscopic Appearance of *B. anthracis* Spores Exposed to Chlorine in the Presence of BSA.

The protective effect of BSA on the *B. anthracis* spore during chlorine exposure was investigated by phase-contrast microscopic examination. Dormant spores exposed to 864-ppm chlorine at 85°C for 2 minutes in the absence of BSA rapidly lysed. In contrast, spores exposed to the chlorine dose in the presence of 1.6-μg/μl BSA exhibited minimal alteration. In the presence of BSA, chlorine-treated spores became somewhat darker and the spore coat thickened.

TABLE 45. Phase-contrast Microscopic Appearance of *B. anthracis* Spores Treated with 864-ppm Chlorine in the Presence and Absence of BSA

BSA (μg/μl)	Microscopic Characteristics
0	Spore lysis, no intact spores, debris
1.6	Intact spores, thickened spore coat, core of most spores somewhat dark

7.1.7. PCR of *B. anthracis* Spores Exposed to an Elevated Concentration of Chlorine in the Presence of BSA.

Phase-contrast microscopic examination of dormant *B. anthracis* spores exposed to a high chlorine residual in the presence of 1.6 μg/μl BSA revealed morphological alteration of the spore by (1) thickening of the spore coat and (2) transition of the spore core from bright to somewhat dark (TABLE 45). This experiment was performed to determine whether spores treated under these conditions could be detected by PCR. Dormant *B.anthracis* spores exposed to 876-ppm chlorine at 85°C for 3 minutes in the presence or absence of BSA were evaluated by PCR amplification of *B. anthracis* target DNA using thermal cycling method 16.

Results. PCR amplification product was not generated from spores exposed to 876-ppm chlorine in the presence or absence of BSA (TABLE 46). Addition of BSA did not prevent PCR interference. Thus, chlorine treatment that yields spores altered morphologically without lysis may generate material that interferes with PCR.

TABLE 46. PCR of *B. anthracis* Spores Exposed to a High Chlorine Concentration with or without BSA

Spore Treatment	PCR Product
Spores + 876-ppm Chlorine	--
Spores + 876-ppm Chlorine + 1.6 μg/μl BSA	--
Spores Alone (Control)	++

7.1.8. PCR of *B. anthracis* Spores Exposed to Chlorine at 85°C in the Presence of a Range of BSA Concentrations.

The presence of 1.6-μg/μl BSA during exposure of *B. anthracis* spores to chlorine appeared to negate generation of material that interfered with PCR (TABLE 42). To determine the amount of BSA required to exert this effect, dormant *B. anthracis* spores (about 5×10^5 per reaction) were exposed to 50-ppm chlorine at 85°C for 1 minute in the presence of 1,600-, 160-, 16-, 1.6-, or 0.16-ng/ml BSA. After addition of 10-mM sodium thiosulfate to neutralize chlorine, the mixtures were evaluated by PCR amplification of *B. anthracis* target DNA using thermal cycling method 16.

Results. Only the highest BSA concentration tested (1,600 ng/μl) negated production of PCR-interfering material generated during chlorine exposure (TABLE 47). PCR amplification of chlorinated spores occurred only in the presence of 1,600 ng/μl BSA (TABLE 47). None of the lower BSA concentrations prevented the PCR inhibition associated with chlorine exposure.

TABLE 47. PCR Amplification of *B. anthracis* Spores Exposed to 50-ppm Chlorine at 85°C for 1 Minute in the Presence of BSA Concentrations Ranging from 0.16 to 1,600 ng/μl

BSA Concentration (ng/μl)	Amplification in Presence of 50-ppm Chlorine
1,600	++
160	--
16	--
1.6	--
0.16	--
0	--

7.1.9. PCR of *B. anthracis* Spores Exposed to Chlorine at 50°C in the Presence of a Range of BSA Concentrations.

Chlorine-mediated disruption of the dormant *B. anthracis* spore proceeded more slowly at 50°C than at 85°C (data not presented). Thus, exposure to lower chlorine residuals could perhaps generate spores devoid of material that interferes with PCR. This experiment was conducted to determine if spores exposed to a lower chlorine concentration in the presence of BSA could yield material suitable for PCR. Dormant *B. anthracis* spores (about 5×10^5 per reaction) were exposed to 50-ppm chlorine at 50°C for 5 minutes in the presence of 1,600-, 160-, or 16-ng/ml BSA. After addition of 10-mM sodium thiosulfate to neutralize chlorine, the mixtures were evaluated by PCR amplification of *B. anthracis* target DNA using thermal cycling method 16.

Results. PCR amplification of chlorinated spores only occurred during treatment in the presence of 1.6-μg/ml BSA (TABLE 48). This is the BSA concentration that negated generation of PCR-interfering material during *B. anthracis* spores exposure to a higher temperature (TABLES 42 and 45).

TABLE 48. PCR Amplification of *B. anthracis* Spores Exposed to 50-ppm Chlorine at 50°C for 5 Minutes in the Presence of a Range of BSA Concentrations

BSA Concentration (ng/ml)	PCR Amplification in Presence of 50-ppm Chlorine
1,600	++
160	--
16	--
0	--

7.2. Dialysis and DNA Purification.

7.2.1. Dialysis to Remove PCR-interfering Material Generated during Chlorination of *B. anthracis* Spores at 50°C.

Experiments were conducted to determine whether PCR-interfering material generated by the interaction of chlorine with the spore was dialyzable. Dormant *B. anthracis* spores (about 1.5×10^6) were treated with 0- or 75-ppm chlorine at 50°C for 5 minutes. An aliquot of the treated spores was dialyzed against 1-L de-ionized water using a microcollodion bag at ambient temperature for 1.3 hour. Another aliquot remained at room temperature without dialysis. PCR amplification of Lambda bacteriophage genomic DNA template using thermal cycling method 16 as described above was conducted in the presence of the two samples.

Results. Substantial inhibition of Lambda bacteriophage DNA amplification occurred in the presence of chlorinated spores with or without dialysis (TABLE 49). Thus, the dialysis treatment did not remove chlorine-generated material that can interfere with PCR.

TABLE 49. PCR Amplification of Lambda Bacteriophage DNA Template in the Presence of Chlorinated Spores with and without Dialysis

Genomic DNA (ng/ml)	Unchlorinated Spores	Chlorinated Spores	Dialyzed Chlorinated Spores	λ Control (No Spores)
10	+++	+	--	+++
1	++	--	--	++

7.2.2. Optimization of *B. anthracis* Spore Lysis by Chlorine Exposure as Evaluated by Microscopic Appearance.

The approach taken thus far to enhance PCR amplification of the *B. anthracis* spore has focused on treatment of spores with chlorine and adding treated spores directly to PCR mixtures for amplification. However, DNA of chlorinated spores was not amplified by PCR. As an another approach, dormant spores were lysed with elevated concentrations of chlorine and then DNA was recovered and purified using commercial DNA purification columns. In this experiment, dormant *B. anthracis* spores were treated with a range of chlorine at 65 or 85^{o}C and examined by phase-contrast microscopy to identify treatment that can promote rapid disruption or lysis of spores (TABLES 50, 51, and 52).

TABLE 50. Phase-contrast Microscopic Appearance of Spores Exposed to Chlorine at 85^{o}C for 1 Minute

Chlorine (ppm)	Spore Appearance Following Chlorine Treatment
0	Intact, refractile
25	Refractile, thickened spore coat
50	All spores dark
100	All spores dark, debris
200	Most spores lysed, no inact spores, much debris

TABLE 51. Phase-contrast Microscopic Appearance of Spores Exposed to Chlorine at 65°C for 5 Minutes

Chlorine (ppm)	Spore Appearance of Following Chlorine Treatment
0	Intact, refractile
25	Many spores ranged from slightly dark to dark
50	No intact spores
100	No intact spores

Table 52. Phase-contrast Microscopic Appearance of Spores Exposed to Chlorine at 65°C for 2 Minutes

Chlorine (ppm)	Spore Appearance Following Chlorine Treatment
0	Intact, refractile
25	About 50% spores ranged from slightly dark to dark, about 50% spores dark
50	Spores ranged from slightly dark to dark
100	Almost all spores dark

7.2.3. GeneClean Column (Bio101) Processing of *B. anthracis* Spores Lysed by Chlorine to Produce Sample DNA without PCR Interference.

Dormant *B. anthracis* spores were treated with 100-ppm chlorine at 85°C for 2 minutes to lyse the organism. Lysis was indicated by reduced turbidity of the spore suspension. In addition, phase-contrast microscopic examination revealed no intact spores and what appeared to be empty spore coats. The lysed spore suspension was processed using the GeneClean Spin column (Bio101) according to the manufacturer's protocols to recover and purify DNA. PCR amplification of *B. anthracis* template using thermal cycling method 16 as described above was conducted with the processed spore DNA sample.

Results. DNA recovered from unchlorinated spores using the GeneClean system yielded material that was amplified by PCR (TABLE 53). However, any material that may have been recovered from the chlorinated spores was not amplified by PCR. Perhaps the GeneClean process did not remove substances that could interfere with PCR or recovery of DNA was low. As another explanation, the DNA recovered from the spores may have been altered by the extensive chlorine treatment and rendered ineffective as template for PCR amplification.

Table 53. PCR Amplification of *B. anthracis* Spore Lysate Processed with the GeneClean Column (Bio101)

Chlorine (ppm)	PCR Amplification
0	++
100	--

7.2.4. DNA Wizard Clean-up System (Promega) Processing of *B. anthracis* Spores Lysed by Chlorine to Produce Sample DNA without PCR Interference.

Dormant *B. anthracis* spores were treated with 100-ppm chlorine at 65°C for 5 minutes to lyse the organism. DNA of the chlorinated spore suspension was processed using the Wizard Clean-up System (Promega) according to the manufacturer's protocols to recover and purify DNA. PCR amplification of *B. anthracis* template using thermal cycling method 16 as described above was conducted with the processed spore DNA sample.

Results. Processing of chlorine-treated or unchlorinated spores with the Wizard Clean-up System did not yield sample material that could be amplified by PCR (TABLE 54). As for the GeneClean system, failure to recover effective PCR template could be attributed to (1) ineffective removal of substances that could interfere with PCR, (2) low recovery of DNA, or (3) alteration of template DNA by the extensive chlorine treatment. Failure to recover PCR-suitable DNA using the Promega system was experienced with other applications in our laboratory.

Table 54. PCR Amplification of Spores Exposed to Chlorine Followed by DNA Purification Using the DNA Wizard Clean-Up System (Promega)

Chlorine (ppm)	PCR Amplification
0	--
100	--

7.2.5. PCR of *B. anthracis* DNA Added to Material Recovered from Chlorinated *B. anthracis* Spores Using the GeneClean Column (Bio101) Sample Processing System.

The GeneClean column (Bio101) sample processing system was not effective for recovering PCR-amplifiable material from dormant *B. anthracis* spores exposed to 100-ppm chlorine at 85°C for 2 minutes (TABLE 53). Since the extensive chlorine exposure may have altered template DNA, spores were exposed to chlorine at a range of lower concentrations (10-, 25-, 50-, and 100-ppm) and lower temperatures (50°C and ambient temperature). Processed DNA samples recovered from the spores were evaluated for effectiveness as PCR template using the PCR system for *B. anthracis* and thermal cycling method 16 as described in an above section. In addition, purified *B. anthracis* DNA (0.2 μl) was added to the preparations (10 μl) to determine if the GeneClean-processed chlorinated spores contained material that could interfere with PCR.

Results. While PCR-amplifiable material was recovered from unchlorinated spores, none of the material recovered from spores chlorinated at ambient temperature or 50°C was suitable for PCR amplification (TABLE 55). However, *B. anthracis* DNA added to GeneClean-processed chlorinated spore material was amplified by PCR (TABLE 56). Thus, the processed material appeared not to contain substances that interfered with PCR. Failure of the GeneClean column system to recover PCR-amplifiable DNA from chlorinated *B. anthracis* spores could be attributed to (1) modification of spore DNA to a non-amplifiable form during the chlorination exposure or (2) inability of the GeneClean system to recover DNA from chlorinated spores. The latter possibility is supported by the fact that ionic charge is the basis of the GeneClean column process and that chlorine reaction with DNA could alter the charge of the nucleic acid.

Table 55. PCR Amplification of Material Recovered from Spores Exposed to Various Chlorine Concentrations and Temperatures Using the GeneClean Purification Column (Bio101)

Chlorine (ppm)	25°C	50°C
0	++	++
10	trace	--
25	trace	--
50	--	--
100	--	--

Table 56. PCR Amplification of *B. anthracis* DNA Amended to Material Recovered from Chlorinated Spores Using the GeneClean Purification Column (Bio101)

Spore Chlorination Conditions	Amplification
10-ppm Chlorine at 50°C	++
50-ppm Chlorine at 50°C	++
100-ppm Chlorine at 50°C	++

7.3 Treatment of *B. anthracis* Spores with Chlorine and Antimicrobial Peptides.

7.3.1. PCR of *B. anthracis* Spores Treated with Protamine and Then Exposed to Chlorine.

Findings of a previous investigation in our laboratory indicated that partially germinated spores were rapidly disrupted by exposure to the polycationic peptide protamine. However, this peptide inhibits PCR in low concentrations (10 ng/ml). This experiment was performed to determine whether chlorine was capable of neutralizing and reversing the PCR inhibition caused by protamine. It was previously determined that chlorine could partially reverse the inhibitory effect that protamine imposed on PCR amplification of Lambda bacteriophage DNA.

In this experiment, dormant *B. anthracis* spores were partially germinated for 15 minutes at 37°C with shaking at 275 rpm. The spores were harvested, suspended in de-ionized water, and

incubated with 0.1-, 1.0-, or 10-μg/ml protamine for 15 minutes at room temperature. The samples were then exposed to 0-, 10-, or 25-ppm chlorine at room temperature and then assayed directly by PCR (5 x 10^5 spores per PCR reaction) using the *B. anthracis* PCR system with thermal cycling method 16 as described in an above section.

Results. Each tested protamine concentration (0.1, 1.0, and 10μg/ml) inhibited PCR (TABLE 57). Thus, chlorine exposure did not neutralize the effect of protamine on PCR. In this experiment, chlorine did not inhibit PCR when the exposure which was performed at ambient temperature. This result is in agreement with the previous finding that chlorination of spores at ambient temperature had little effect on the microscopic appearance of dormant spores. However, at elevated temperatures chlorine exposure altered the microscopic appearance of the spore coat and generated PCR interference.

Table 57. PCR Amplification of *B. anthracis* Spores Treated with Protamine and Then Exposed to Chlorine

Protamine (μg/ml)	0-ppm Chlorine	10-ppm Chlorine	25-ppm Chlorine
0	++	++	++
0.1	--	--	--
1	--	--	--
10	--	--	--

7.3.2. PCR of *B. anthracis* Spores Treated with Chlorine and Then Exposed to Gramicidin S.

This experiment was conducted to determine whether treatment with chlorine and gramicidin S could enhance PCR-based detection of dormant *B. anthracis* spores. Spores were chlorinated with either 2- or 10-ppm chlorine for 2 minutes at 85°C. The chlorine was neutralized with 10-mM sodium thiosulfate and then 130-μg/ml gramicidin was added. The samples were incubated at room temperature for 20 minutes and then assayed by PCR using the *B. anthracis* PCR system with thermal cycling method 16 as described in an above section.

Results. Spores treated with chlorine and gramicidin S yielded DNA material that could be amplified by PCR. However, the quantity of PCR product was less than amount generated with chlorine treatment alone (TABLE 58). Phase-contrast microscopic examination of spores that were first treated with 2- or 10-ppm chlorine and then exposed to 130-μg/ml gramicidin revealed thickening of the spore coat and generation of debris.

Table 58. PCR Amplification of *B. anthracis* Spores Treated with Chlorine and Then Exposed to Gramicidin S

Chlorine (ppm)	Chlorine, No Gramicidin	Chlorine, 130-μg/ml Gramicidin
0	++	+
2	++	+
10 ppm	--	--

7.4. Treatment of Chlorinated *B. anthracis* Spores with Reducing Agents.

7.4.1. PCR of *B. anthracis* Spores Exposed to Chlorine and Then Treated with Cysteine to Neutralize Chlorine.

If exposure of *B. anthracis* spores to chlorine generates materials that interfere with PCR as the result of oxidation reactions, then reversal of the interference may be possible by treatment with reducing agents. It has been reported that adenosine and nucleotides react with hypochlorite to form unstable products that have been identified as the N6 chloramine derivatives. These chloramines spontaneously oligomerize forming stable adducts with proteins and nucleic acids. In addition, reducing agents can convert the chloramines to the original nucleoside or nucleotide with the loss of chlorine (Free Radic. Res. Commun. 1990. 9:303-315).

Cysteine was evaluated as a reducing agent to treat spores following chlorination. In this experiment, dormant *B. anthracis* spores (5×10^5 per reaction) were exposed to 0-, 25-, or 50-ppm chlorine at 85°C for 1 minute. Each treatment was amended with 0, 0.01, or 0.02% cysteine to neutralize the halogen and then assayed by PCR using the *B. anthracis* PCR system with thermal cycling method 16 as described in an above section.

Results. Cysteine treatment of chlorinated spores did not negate the effects of the halogen on PCR (TABLE 59).

Table 59. PCR Amplification of Chlorinated *B. anthracis* Spores in the Presence of the Cysteine as a Reducing Agent

	Chlorine (ppm)		
Cysteine (Percent)	**0**	**25**	**50**
0	++	--	--
0.01%	++	--	--
0.02%	++	--	--

7.4.2. PCR of *B. anthracis* Spores Exposed to Chlorine and Then Treated with Dithiothreitol.

Dithiothreitol was evaluated for its potential as a reducing agent that could reverse the effect of chlorine exposure on PCR of *B. anthracis* spores. Incorporation of 10-mM dithiothreitol in PCR mixtures could permit PCR amplification of DNA in boiled lysate that could not be amplified under standard conditions (M. Nagai, A. Yoshida, and N. Sato. Additive effects of bovine serum albumin, dithiothreitol, and glycerol on PCR. 1998. Biochem. Mol. Biol. **44:**157-163).

In this experiment, dormant *B. anthracis* spores (5×10^5 per reaction) were exposed to 0-, 25-, or 50-ppm chlorine at 85°C for 1 minute. After neutralizing chlorine with 10-mM sodium thiosulfate, the samples were treated with 5-, 10-, or 20-mM dithiothreitol for 20 minutes at room temperature. When the samples were added to PCR mixtures, dithiothreitol final concentrations of the PCR mixtures were to 1.6, 3.2 and 6.3 mM. The mixtures were assayed by PCR using the *B. anthracis* PCR system with thermal cycling method 16 as described in an above section.

Results. While dithiothreitol did not affect PCR amplification with unchlorinated spores, the compound did not negate the PCR interference caused by reactivity of chlorine with spores (TABLE 60).

Table 60. PCR Amplification of Chlorinated *B. anthracis* Spores Treated with Dithiothreitol

	PCR Mix Dithiothreitol (mM)		
Chlorine (ppm)	**1.6**	**3.2**	**6.3**
0	++	++	++
25	--	--	--
50	--	--	--

7.4.3. PCR Amplification of *B. anthracis* Spores Exposed to Chlorine and Then Treated with Elevated Concentrations of Dithiothreitol.

The potential of higher levels of dithioithreitol to reverse the PCR interference associated with chlorine-exposed spores was examined. Dormant *B. anthracis* spores were exposed to 0- or 25-ppm chlorine at 85°C for 1 minute prior to treatment with elevated dithiothreitol levels for 20 minutes at ambient temperature. Final dithiothreitol concentrations in PCR mixtures were 5-, 10-, and 20-mM. The mixtures were assayed by PCR using the *B. anthracis* PCR system with thermal cycling method 16 as described in an above section.

Results. Dithiothreitol concentrations ranging from 5- to 20-mM in the PCR mix did not affect PCR amplification with unchlorinated spores. However, the reducing agent did not reverse the PCR interference of the chlorination spores (TABLE 61).

TABLE 61. PCR Amplification of Chlorinated *B. anthracis* Spores Treated with Elevated Levels of Dithiothreitol

	PCR Mix Dithiothreitol (mM)			
Chlorine (ppm)	**0**	**5**	**10**	**20**
0 ppm	++	++	++	++
25 ppm	ND	weak	weak	--

8. CHLORINE CONSUMPTION BY *B. ANTHRACIS* SPORES.

A series of experiments was performed to quantify chlorine consumption by purified dormant spores. Chlorine consumption experiments were performed in accordance to the protocols that had been used to disrupt dormant spores by chlorine exposure at an elevated temperature. The procedure involved (1) suspending spores (from 1×10^6 to 5×10^7) in 10 ml chlorine solution, (2) heating the suspension at 85°C for 2 minutes, (3) cooling the suspension to ambient temperature, (4) removing spores from the liquid using a membrane filter (0.22-μm pore diameter), and (5) measuring the chlorine residual of the filtrate using the DPD (N,N-diethyl-p-phenylenediamine) colorimetric method. As a comparative control, the procedure included chlorine solution with no spores.

Results. Compared with the control lacking spores, chlorine consumption was demonstrated with each spore suspension (Table 62). The level of chlorine consumption increased with the number of spores exposed to the halogen. However, a linear relationship between the number of chlorine molecules consumed per spore and the number of spores exposed was not demonstrated (Table 62 and Figure 6). As spore number or density increased, the number of chlorine molecules consumed per spore decreased.

TABLE 62. Chlorine Consumption by *B. anthracis* Spores during Chlorine Exposure at 85°C for 2 Minutes

Number of Spores	Chlorine (ppm) after 85°C for 2 Minutes	Reduction in Chlorine (ppm) Due to Spores	Chlorine Molecules Consumed by Spores	Chlorine Molecules Consumed Per Spore
0 (control)	**0.84**	**0**	**0**	**0**
1×10^6	**0.71**	**0.13**	**2.2×10^{16}**	**2.2×10^{10}**
5×10^6	**0.57**	**0.27**	**4.6×10^{16}**	**9.2×10^9**
1×10^7	**0.52**	**0.32**	**5.4×10^{16}**	**5.4×10^9**
5×10^7	**0.11**	**0.73**	**1.2×10^{17}**	**2.5×10^9**

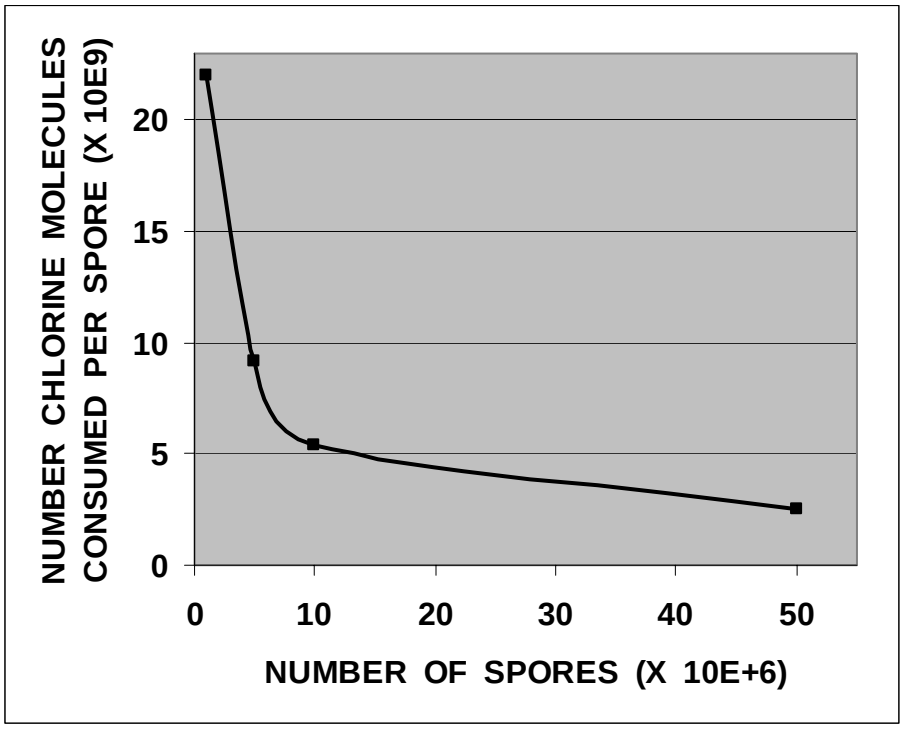

FIGURE 6. Effect of spore number or density on chlorine consumption per *B. anthracis* spore during chlorine exposure at 85°C for 2 minutes.

9. SUMMARY OF INVESTIGATION.

PCR can provide the basis for rapid and reliable detection of microbial pathogens. Amplification of a DNA sequence that is unique for a target microbial species would allow accurate detection of the organism following a short testing time. In addition, PCR offers potential for sensitive detection since only a few copies of target DNA are required to generate a significant detection signal. Sensitive PCR-based detection of a variety of bacterial species, including *Legionella pnuemuphila, Escherichia coli* serotype O157:H7, and *Vibrio cholerae*, has been demonstrated in our laboratory. As few as ten cells of each species delivered directly to a PCR mixture contained in a reaction tube yielded robust detection signal. Because sensitive detection was accomplished using intact organisms, it appeared that treatment of cells was not required for effective PCR detection of bacterial species. Perhaps elevated temperatures during PCR thermal cycling was sufficient to disrupt the bacterial cell wall and allow DNA to be accessible for PCR. In contrast, PCR detection of *B. anthracis* spores was not sensitive. About one thousand dormant anthrax spores placed in a PCR mixture was required to generate a significant detection signal. This observation could be attributed to the recalcitrance of the spore wall to antagonistic conditions, such as elevated heat, and the ability of the outer structure to maintain its barrier properties. Consequently, an objective of this study was to derive a treatment that could render nucleic acids within the bacterial spore more assessable for amplification by PCR. As a common disinfectant known to disrupt the wall of microorganisms, chlorine was investigated for its potential use in treating the *B. anthracis* spore for more effective PCR-based identification.

Chlorine could alter *B. anthracis* spore wall integrity through reaction of the halogen with biomolecules of the spore's outer structure. Chlorine reactivity with the spore wall could lead to disruption of the wall barrier and release of materials such as nucleic acids from the spore. Thus, the central objective of this study was to investigate the potential of chlorine treatment for releasing DNA from the *B. anthracis* spore as a means to enhance PCR-based detection of the pathogen. While it was assumed that the primary action of chlorine would be associated with the spore wall, it was also considered that chlorine treatment could alter released DNA. Although earlier studies in our laboratory indicated that DNA exposed to chlorine remained suitable as template for PCR, severe chlorine treatments could alter the nucleic acid and reduce its function for PCR. Thus, this investigation focused on understanding how chlorine treatment could (1) alter spore wall integrity and, thereby, promote release of DNA and (2) affect DNA as PCR template. Optimal chlorine treatment would promote release of DNA from the spore while not affecting the nucleic acid as PCR template.

As indicated in this study through microscopic examination, chlorine treatment at an elevated temperature, such as 50 or 85^{o}C, for a short period significantly altered the dormant *B. anthracis* spore. Alternately, exposure to chlorine at ambient temperature appeared not to impose any dramatic effect on dormant spore morphology. Treatment with chlorine at an elevated temperature promoted morphological alterations that typically occur during spore activation and initial stages of germination. The morphological alterations as observed by phase-contrast microscopy included (1) thickening of the spore wall and (2) darkening of the spore interior. These alterations during activation and germination reflect profound changes in the spore wall as the organism transforms from a spore to a cell. Concurrent with these changes would be increased permeability of the organism's wall and, consequently, sensitivity to antimicrobial compounds. This was observed earlier in our laboratory during an investigation of the effect of protamine on the dormant *B. anthracis* spore. While dormant spore morphology and permeability appeared not to be affected by exposure to high levels of the protamine at ambient temperature, exposure to the compound at elevated temperatures yielded spores with darkened interior and increased permeability to propidium iodide. Alteration of the dormant bacterial spore upon exposure to a compound at an elevated temperature was also observed when examining the effect of dodecylamine on *B. subtilis* (B. Setlow, A. E. Cowan, and P. Setlow. 2003. Germination of spores of *Bacillus subtilis* with dodecylamine. J. Appl. Microbiol. **95:**637-648). Researchers of the study suggested that exposure of the compound promoted spore germination. However, since the treated spores did not elongate and transform to cells, the effect was, in effect, an incomplete or lethal germination.

Our study focused on investigating treatment with chlorine at elevated temperatures for its potential to enhance DNA-based detection of the dormant *B. anthracis* spore. The treatment could constitute a simple and rapid means to render DNA within the dormant spore accessible for efficient amplification by PCR. As indicated by microscopic examination, the treatment significantly altered the dormant spore. Since the alterations reflected events that could be associated with increase wall permeability, the treatment would be expected allow release of DNA and, thus, promote PCR based detection. However, chlorine treatment of dormant *B. anthracis* spores appeared to decrease PCR detection sensitivity. It was considered that the observed effects of chlorine treatment on PCR could be attributed to chlorine-induced alteration of DNA or release of spore materials that interfere with PCR. To understand the effect of chlorine treatment on PCR-based detection of anthrax spores and modify the treatment to enhance PCR, the investigation focused on elucidating the effects of chlorine exposure on (1)

structural integrity of purified DNA species, (2) suitability of purified DNA species as PCR template, and (3) generating spore material that could interfere with PCR. A central approach to investigating these factors was relating the ratio of reactive chlorine molecules to target as DNA species or spore.

In this study, purified DNA species appeared to maintain structural and functional integrity following exposure to substantial levels of chlorine. For exposure of various quantities of DNA to various doses of chlorine, the general trend was that the biomolecule could maintain integrity when exposed to about 50 chlorine molecules per DNA base-pair. This was illustrated by gel electrophoresis and ethidium bromide staining to visualize purified DNA species following chlorine exposure. For PCR product exposed to chlorine at 85^{o}C for one minute, no effect on DNA band intensity or electrophoretic mobility was observed with a chlorine molecule to DNA base-pair ratio of 72 for Lambda bacteriophage target and 56 for *B. anthracis* target. Minimal effects on structural integrity (slight smearing of DNA band) after chlorine exposure at 85^{o}C for one minute were observed with a chlorine molecule to DNA base-pair ratio of 143 for Lambda bacteriophage target and 112 for *B. anthracis* target. Evaluation with *Hin*dIII-digested Lambda bacteriophage DNA fragments indicated no effect following chlorine exposure at 85^{o}C for one minute at a chlorine to base pair ratio of 45. However, exposure to a ratio of 67 or 89 yielded DNA fragments with increased apparent molecular weight and no decrease in DNA band intensity. This observation could be attributed to addition reactions of chlorine with nitrogen groups of DNA. Such reactions would increase DNA fragment mass and alter electrophoretic mobility. Exposure of *Hin*dIII-digested Lambda bacteriophage DNA to elevated chlorine doses (112, 134, or greater than 1,117 chlorine molecules per DNA base pair) generated material that was not visible. Perhaps exposure to very high chlorine levels resulted in substantial reaction with the DNA nitrogen groups involved with hydrogen bonding between base pairs. Disruption of base pair interactions could denature DNA to single-stranded structures that would not stain with ethidium bromide.

Tolerance of purified DNA species to chlorine exposure was also illustrated in this study by examining how the halogen affected the ability of DNA to serve as PCR template. Compared with control DNA not exposed to chlorine, Lambda bacteriophage PCR product exposed to chlorine at 85^{o}C for one minute at a ratio of 72 chlorine molecules per DNA base pair appeared to be unaltered with respect to effectiveness as PCR template. For exposure of *B. anthracis* PCR product to chlorine at 85^{o}C for 1 minute, a ratio of 56 chlorine molecules per DNA base pair appeared not to affect the DNA as PCR template. Exposure of *B. anthracis* PCR product at a ratio of 112 chlorine molecules per DNA base pair exerted a partial effect on the DNA as PCR template.

Because pure DNA species maintained integrity upon exposure to high doses of chlorine, it appeared that conditions of chlorine treatment could be derived for optimal disruption of the dormant *B. anthracis* spore and release of DNA for effective PCR-based detection. However, chlorine exposures that disrupted the dormant spore, as determined by microscopic examination, did not enhance PCR signal. In fact, the general trend was that chlorine treatments that altered spore morphology also diminished PCR signal. This effect could be attributed to generation of material that interfered with PCR. Results of this study indicated that chlorination of *B. anthracis* spores at elevated temperatures appeared to have generated material that interfered with PCR. PCR of Lambda bacteriophage template DNA was inhibited when conducted in the presence of *B. anthracis* spores that were previously exposed to 25-ppm chlorine at 50^{o}C for 5 minutes and then amended with sodium thiosulfate to neutralize the halogen. Inhibitory material

appeared to be associated with the spores. PCR was inhibited when conducted in the presence of chlorinated spores that were recovered with a filter to eliminate the suspension liquid. In contrast, PCR was not affected in the presence of the spore-free filtrate of the chlorinated spores. In addition, PCR was inhibited in the presence of chlorinated spores that were washed by dialyzing against de-ionized water.

The effect of chlorine treatment at elevated temperatures on promoting the release of DNA from *B. anthracis* spores was difficult to elucidate in this study. Alteration of the dormant spore wall and enhancement of permeability by the treatment was suggested by (1) conversion of refractive spores to dark spores as observed by phase-contrast microscopy and (2) uptake of propidium iodide as observed by epifluorescent microscopy. However, while enhanced permeability of the spore wall could allow release of DNA from the spore and, thereby, improve PCR-based detection, release of DNA from the spore could not be demonstrated. The number of spores that would be required to yield a sufficient quantity of DNA to be observed by gel electrophoresis and ethidium bromide staining greatly exceeded the number that could be tested. In addition, release of plasmid DNA from chlorine-treated spores was not demonstrated by PCR analysis of spore-free filtrate.

A major obstacle to employing treatment of *B. anthracis* spores with chlorine at elevated temperatures as a means to enhance PCR-based detection appeared to be generation of material that inhibited PCR. Even if DNA was released from the spore and remained intact as PCR template, the presence of PCR inhibitors would impede effective DNA-based detection. The inhibitory material appeared to be intimately associated with the spore since treated spores remained inhibitory following washing or dialysis. Perhaps tight binding of inhibitory material with the spore surface may have prevented removal of the material by wash treatments. As another possibility, inhibitory material residing within the spore may have been released from the spore during PCR as a result of the high temperatures of the thermal cycles. Inhibitors released from spores during thermal cycling could adversely affect PCR through interaction with *Taq* DNA polymerase, Mg^{2+} cations, DNA template, primers, or other components that are crucial to PCR. Release of material from chlorine-treated *B. subtilis* spores as a result of subsequent thermal treatment has been reported (S. B. Young and P. Setlow. Mechanisms of killing of *Bacillus subtilis* spores by hypochlorite and chlorine dioxide. J. Appl. Microbiol. 2003. **95:**54-67). While *B. subtilis* spores treated with chlorine did not release dipicolinic acid (DPA) from the spore cortex, subsequent exposure of the spore to sub-lethal elevated temperatures promoted release of the material. DPA could interfere with PCR. As an ionic compound that readily complexes with Ca^{+2} in the spore, DPA could interact with Mg^{2+} in PCR mixtures and thereby adversely affect the activity of the *Taq* DNA polymerase. Release of DPA during PCR and consequent inhibition of PCR could explain our observations on generation of PCR inhibitors during chlorine treatment of *B. anthracis* spores at elevated temperatures. Failure of washing or dialysis treatment at ambient temperature to remove DPA from treated spores could have resulted from DPA residing within the spore and not being accessible for removal. Perhaps washing or dialysis at an elevated temperature could have been effective if DPA were released from the spore and then removed by the treatment.

In addition to chlorine treatment, exposures to other oxidants have been reported to modify the *B. subtilis* spore so that the treated organism could release DPA upon subsequent exposure to heat. While dormant *B. subtilis* spores killed by exposure to peroxynitrite retained DPA, the compound was not retained by the treated spores upon heat treatment (P. C. Genest, B. Setlow, E. Melly, and P. Setlow. Killing of spores of *Bacillus subtilis* by peroxynitrite appears

to be caused by membrane damage. Microbiol. 2002. **148:**307-314). Similar observations were reported for *B. subtilis* spores exposed to superoxidized water (C. A. Loshon, E. Melly, B. Setlow, and P. Setlow. Analysis of the killing of spores of *Bacillus subtilis* by a new disinfectant, Sterilox®. J. Appl. Microbiol. **91:**1051-1058). Perhaps oxidizing disinfectants exerted a similar mechanism for spore alteration and thermal-inducible release of DPA. For the work conducted with spores exposed to peroxynitrite, researchers proposed that the oxidant generated severe permeability defects in the spore inner membrane. While these defects were evident by uptake of propidium iodide, the membrane retained the ability to contain DPA. Perhaps the inner membrane was modified by oxidant exposure so that subsequent heat exposure would damage the structure to the extent that DPA could exit the spore core.

The findings of our study illustrated the problem of employing chlorine treatment at elevated temperatures as a means to enhance DNA-based detection of the *B. anthracis* spore. While the treatment may be effective for rapid alteration of the dormant spore wall to allow DNA confined within the spore to become accessible for PCR, the treatment appeared to release material that interfered with PCR. Consequently, the chlorine treatment procedure requires modification to overcome the effect of interfering material. The Electro-Fractionation System (EFS), which was developed in our laboratory, could be employed for effective PCR of the spore. The EFS employs electric current to promote migration of nucleic acids from complex materials as a means to recover nucleic acids from complex materials that interfere with PCR. The EFS was found to be effective for recovering Lambda bacteriophage genomic DNA from a variety of complex matrices, including turbid river water, turbid hot tap water, and various turbid liquid foods. In addition, Lambda bacteriophage DNA recovered from turbid river water using the EFS was effective as template for PCR. Thus, the EFS offers potential for both recovery and cleansing of nucleic acids associated with complex samples. If DPA were released from the *B. anthracis* spore during chlorine treatment at elevated temperature, then the EFS could effectively dissociate the compound from spore DNA since the EFS removes compounds that are smaller than DNA. Assessment of chlorine treatment at elevated temperature with the EFS as a means for effective detection of the *B. anthracis* spore would require evaluation of the two systems conducted together. In addition, to understand the generation of material that can interfere with DNA-based detection, it should be determined whether or not (1) DPA is released from *B. anthracis* spores treated with chlorine and heat and (2) DPA inhibits PCR.